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THE GENETIC BIOASSAY BRANCH PROTOCOL FOR BACTERIAL MUTAGENESIS INCLUDING SAFETY AND QUALITY ASSURANCE PROCEDURES

by

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Researchers often choose microbial tests for use in genetic toxicology studies because they are rapid and inexpensive, and because they supply dose-responsive data. This user's manual documents the test methods used extensively by the Health Effects Research Laboratory (HERL), Research Triangle Park, North Carolina over the past four years.

The first part of the report describes the basic protocol (the plate incorporation test), special precautions, and specific modifications of the assay routinely used in HERL studies. The second part presents HERL's stepwise protocol for the plate incorporation test and protocols for several other biological assays, including the spot test, the forward mutation assay procedure, and the fluctuation test. The last portion of the report provides the necessary information for experiment planning and provides sample information forms and computerization procedures necessary for reporting experimental results.

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FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory participates in the development and revision of air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is primarily responsible for providing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This user's manual provides an overview of bacterial mutagenicity protocols and the detailed protocol for the *Salmonella* plate incorporation assay (Ames test) used by the Health Effects Research Laboratory. In addition, it discusses quality assurance, data computerization, and data interpretation methods used by the Laboratory.

F. G. Hueter, Ph.D.
Director
Health Effects Research Laboratory

ABSTRACT

Researchers often choose microbial tests for use in genetic toxicology studies because they are rapid and inexpensive, and because they supply dose-responsive data. This user's manual documents the test methods used extensively by the Health Effects Research Laboratory (HERL), Research Triangle Park, North Carolina over the past 4 years.

The first part of the report describes the basic protocol (the plate incorporation test), special precautions, and specific modifications of the assay routinely used in HERL studies. The second part presents HERL's stepwise protocol for the plate incorporation test and protocols for several other biological assays, including the spot test, the forward mutation assay procedure, and the fluctuation test. The last portion of the report provides the necessary information for experiment planning and provides sample information forms and computerization procedures necessary for reporting experimental results.

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PART I

OVERVIEW OF PROTOCOLS AND SAFETY PROCEDURES

SECTION 1

INTRODUCTION

A variety of short-term genetic bioassays are currently available for mutagenicity testing of chemical compounds. From these assays, the microbial plate incorporation tests are often chosen for use in genetic toxicology studies because they are rapid and inexpensive, and because they supply dose-responsive data. However, some of the basic protocols for these tests cannot determine mutation frequencies.

Part I of this report describes the basic protocol (the plate incorporation test), special precautions, and specific modifications of the assay routinely used in Health Effects Research Laboratory (HERL) studies. This portion of the report also describes the applications of this assay to complex mixtures such as industrial and mobile source pollutants, and presents an overview of methods for chemically extracting and concentrating pollutant samples.

Part II presents HERL's stepwise protocol for the plate incorporation test as well as protocols for several other biological assays, including the spot test, the forward mutation assay procedure, and the fluctuation test.

The last portion of the report, Part III, provides information necessary for experiment planning and includes sample information forms and computerization procedures necessary for reporting of experimental results.

SECTION 2

BASIC *SALMONELLA TYPHIMURIUM* PLATE INCORPORATION TEST

The reference protocol for the *Salmonella typhimurium* plate incorporation test is described by Ames *et al*. (1). Part II gives the stepwise procedure used by the author of this report. The outline of the basic procedural protocol is very simple (Figure 1-1). A specific amount of the test chemical and a culture of the indicator bacterial strain are added into a melted soft agar overlay. The mixture is then poured onto a minimal media plate that will select for bacteria having undergone mutation. The plate is incubated 48 to 72 h at 37°C and the number of colonies per plate then counted. This procedure is repeated for each dose of the chemical, for each bacterial strain, for each routine modification (e.g., with and without microsomal activation), for each needed control, and for each replication. The procedure generates approximately 210 data points (plate counts) for each chemical analyzed.

The *Salmonella* procedure detects reverse mutations that occur in histidine-requiring strains of *Salmonella typhimurium* developed by Dr. Bruce Ames (1). When these strains undergo a reverse mutation to prototrophy, the revertants can be detected on a minimal nutrient medium that lacks histidine. Although the reverse mutation system detects only reverse mutations, forward mutation detection systems are not limited to a specific type of genetic event

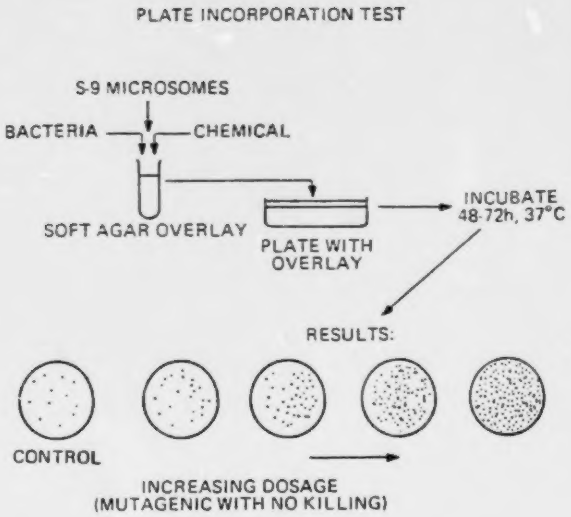
PLATE INCORPORATION TEST
S-9 MICROSOMES
BACTERIA
CHEMICAL
SOFT AGAR OVERLAY
PLATE WITH OVERLAY
INCUBATE 48-72h, 37°C
RESULTS:
CONTROL
INCREASING DOSAGE
(MUTAGENIC WITH NO KILLING)

Figure 1-1. Outline of the basic Salmonella typhimurium plate incorporation test with mammalian activation.

(e.g., a frameshift or a point mutation). Since screening systems need to identify a variety of genetic mutational events, several different Salmonella strains have been developed. Each strain detects a specific type of mutation and incorporates other changes needed to increase its sensitivity. Five strains are commonly used (Table 1-1), three of which (TA1537, TA1538, and TA98) detect frameshift mutations. TA1537 detects compounds such as 9-aminoacridine. TA1538 and TA98 both detect compounds such as 2-nitrofluorene, but differ in that TA98 contains an R-factor plasmid and is more sensitive to some frameshift mutagens than TA1538. Chemicals that produce base-pair substitutions, especially alkylating agents, are detected in TA1535. Strain TA100 was developed from TA1535 by the addition of the R-factor plasmid.

The R-plasmid causes a higher spontaneous mutation rate and can detect chemicals that normally yield frameshift mutations.

TABLE 1-1. CHARACTERISTICS OF FIVE SALMONELLA TYPHIMURIUM TESTER STRAINS

Strain	his-Mutation*	rfa†	uvr B‡	R-factor plasmid	Spontaneous revertants/plate
TA1535	his G46	Yes	Yes	No	5-50
TA1537	his C3076	Yes	Yes	No	2-25
TA1538	his D3052	Yes	Yes	No	5-40
TA98	his D3052	Yes	Yes	Yes	15-35
TA100	his G46	Yes	Yes	Yes	80-200

*his-Mutation: Histidine mutation incorporated
†rfa: Mutation for deficiency in bacterial cell wall
‡uvr B: Ultraviolet repair gene B deletion

In addition to the histidine locus mutation, these *Salmonella* strains carry an *rfa* mutation that creates a deficiency in the bacterial cell wall lipopolysaccharides. This deficiency increases the cell's permeability to large molecules. The *uvr* B mutation was incorporated into these strains to increase their susceptibility to several classes of mutagens by decreasing genetic repair. The R-factor plasmid found in strains TA98 and TA100 increases further the sensitivity of the tester strains by participating in a type of error-prone repair. This plasmid is detected by ampicillin resistance. The five strains also all differ in the number of spontaneous revertants per plate generally found. Table 1-1 summarizes the general characteristics of the strains.

SECTION 3

MAMMALIAN METABOLIC ACTIVATION

Since some compounds that are carcinogenic for mammals are not mutagenic for bacteria, carcinogenic potential in humans cannot always be predicted by bacterial systems. Therefore, a mammalian metabolic activation system was added to the basic Salmonella assay to allow for some of the metabolites as well as the agent itself to be tested for mutagenicity. The activation system described by Ames (1) is usually used and consists of a 9000 x g supernatant of a rat liver homogenate. Generally, Aroclor is administered to the rats from which the homogenate is prepared to induce the hepatic enzymes referred to collectively as mixed function oxygenases (MFO). An induced MFO system potentiates increased metabolite levels and a corresponding increase in revertant colonies due to mutagenic metabolites.

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SECTION 4

PRECAUTIONS AND PROCEDURAL MODIFICATIONS

Although the reference protocol (1) has been described in great detail, protocols with modifications and differing quality control procedures have been presented in the literature as "standard protocols" (2, 3). The modifications and quality assurance procedures used by the author of this report are presented, as closely as possible, in the sequence encountered in the testing protocol.

CHECKING OF TESTER STRAINS

Upon their receipt and at regular intervals, the tester strains are checked for ultraviolet (UV) sensitivity, ampicillin resistance, and crystal violet killing. If these parameters indicate a mixed culture or give an unacceptable response, new single colony isolates are grown from a stock culture tube. The strains are also checked for spontaneous and induced mutant levels at the time of creating master plates.

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CRITERIA FOR PROPER CULTURE

Bacterial strains, culture media, and testing conditions may vary between culture preparations; the bacterial cultures used for testing vary accordingly. Three conditions, however, are always met for the routine screening of chemicals. First, a single colony isolate is always used to initiate the culture. This helps to insure genetic uniformity of the broth culture. Secondly, the test culture always has a concentration of 1-2 x 10^9 viable cells per mL. This concentration is assured by using the culture in the early portion of the stationary growth phase. Next, if the cells are not used immediately upon harvesting, they are stored in an ice water bath.

MAMMALIAN ACTIVATION SYSTEM

This protocol uses the 9000 x g supernatant (S-9) obtained from rat liver induced with Aroclor 1254. This S-9 mixture is prepared by the supplier from Charles River CD-1 rats weighing between 180 and 200 g, using the preparation procedures of Ames (1). The protein concentration is determined by the method of Lowery (4) and recorded. Since the level of enzymatic activity can vary with each batch of S-9, various concentrations of the S-9 are tested within the bioassay against standard concentrations of two or three known mutagens. For each mutagen, the S-9 concentration that provides the maximum number of revertants per plate is determined; the concentrations of the two maximum responses are averaged to provide the S-9 concentration used for testing. The three mutagens used are benzo(a)pyrene, 2-anthramine, and 7,12-dimethylbenzanthracene.

SUBSTANCE TESTED

In order to assure proper testing, several guidelines are followed. Each sample is tested at a minimum of five doses at half-log intervals to survey a broad range of doses. Compounds that demonstrate little or no toxicity are tested in the 5 to 10 mg per plate range before being reported as negative. The solubility of the test substance is noted both when placed into the solvent and into the agar overlay. Agents which precipitate or demonstrate an apparent loss of highly volatile substances are retested with modified conditions. Although more refined procedures are being developed, volatile substances presently are tested in sealed bags or desiccators. Indication of the toxicity is recorded, and if clearing of the test plates is seen at all doses, lower doses are tested to assure that the effect observed is not due to toxicity. When toxicity does result, some of the bacteria may lyse and contribute histidine to the media. Therefore, cross-feeding may result and colonies that are not revertants may be found. These colonies are usually small pinpoint colonies, though not always. Other substances, especially complex mixtures and technical grade chemicals, may contain either histidine or histidine-like acting components. Colonies from representative plates are transferred to minimal media plates without histidine to determine whether or not they are true revertant colonies.

WITHIN EXPERIMENT CONTROLS

All tests contain appropriate positive, negative, and sterility controls. Table 1-2 records positive controls for the five commonly used tester strains.

The negative control consists of the entire system without the compound to check spontaneous mutation rate. The activation mix and test substance are both checked for sterility.

TABLE 1-2. CONTROL MUTAGENS FOR THE FIVE COMMONLY USED TESTER STRAINS OF SALMONELLA TYPHIMURIUM

Strain	Without activation (-S-9)	With activation (+S-9)
TA1535	MNNG, or sodium azide, or methylmethane sulfonate	2-Anthramine
TA1537	9-Aminoacridine	2-Anthramine
TA1538	2-Nitrofluorene, or 4-nitro-o-phenylenediamine	2-Anthramine
TA98	2-Nitrofluorene, or hycanthone methanesulfonate	2-Anthramine
TA100	MNNG, or sodium azide, or MMS, or nitrofurantoin	2-Anthramine

SPECIAL PROCEDURES FOR COMPLEX MIXTURES

Complex mixtures present a variety of technical difficulties in the Salmonella test. Most complex mixtures are direct environmental pollutants which are sampled in various ways. Some of these samples are very low in organics, while others are entirely organic.

The mutagenic activity of a complex mixture may not be detectable because of problems such as too high a dilution, presence of a sample matrix which prevents release of the mutagens into the bacterial media (as in the case with certain carbonaceous particulate samples), or the presence of highly toxic compounds in the mixture which result in sufficient bacterial killing to prevent detection of bacterial mutants. To overcome these problems, most of the complex mixtures tested are concentrated, extracted, or fractionated prior to bioassay. Examples of applications of these methods are described below.

Concentration

Various methods are used to concentrate the pollutant samples. If the sample is in particulate or precipitate form, filtration is used to concentrate it. The type of filtration used depends upon the type of sample. For example, air particulates are generally filtered by the use of pallflex filters and glass fiber filters, and are concentrated by impaction devices and electrostatic precipitation.

Extraction

With particulates, the active organic compounds condense onto a variety of materials including silica and carbon. Since bacteria cannot ingest most of these solid materials (as some mammalian cells are able to), these organics are extracted from the solid matter. Samples for these studies are extracted by a variety of processes (e.g., in a Soxhlet apparatus at 45°C with dichloromethane). Particle mass is determined both before and after extraction, and the percent of organic mass per particle mass is calculated. The dichloromethane-organic extract mixture is blown to dryness or near dryness under dry nitrogen. After weighing, the organics are dissolved in dimethyl sulfoxide (DMSO) or some other appropriate solvent. All samples are tested in a solvent compatible with the biological test system.

SAFETY PRECAUTIONS

The following is a listing of some of the safety procedures established within the author's laboratory:

- All samples for testing and positive control chemicals are housed in a special weighing and storage area. These samples and chemicals are recorded and logged in and out.
- All weighings and measurements of stock chemicals are handled in a special weighing room within glove boxes which are vented to the outside through charcoal filters located on the roof (see Part II, Section 1).
- Whenever possible, incubators, storage cabinets, and refrigerators are vented, and upon opening any of these units, the air within the unit is exhausted.
- All work is performed in laminar flow exhaust hoods or total exhaust biological hoods (see Part II, Section 1).
- All work is completed in designated areas, and personnel are properly clothed. (Proper clothing includes a disposable gown or jump suits, two pair of latex gloves, safety glasses, safety shoes, and shoe covers. Each employee is instructed in the proper use of the clothing.)
- No mouth pipetting is allowed for any procedure.
- No eating, drinking, smoking, chewing of gum, or application of cosmetics is allowed within the laboratory. No food or drink may enter the laboratory.
- If an EPA safety official or the supervisor determines the noise level high enough to warrant ear protection, an ear protection device is worn by all personnel at any time at which the noise reaches this level (e.g., when hoods are operating).
- Each phone is posted with the emergency number (#2900) and the room number in which the phone is located. A copy of the emergency reporting procedure is also adjacent to each phone.

- Each employee must learn and follow all emergency evacuation procedures.
- All waste is double-bagged and placed in drums or special containers which are sealed and sent by a contractor to special areas for disposal or incineration.

DATA INTERPRETATION

Information on controls (both positive and negative), sterility when contamination is reported, and the replication of plates at each dose and replication of each test is either reported for each sample or can be assumed to be within normal limits. This is also true for the number and range of doses used and the toxicity of the sample.

The determination of definite positives in the author's laboratory is based on the following criteria (Statistical tests are based upon the model by Stead *et al*. [5]):

- The data must not vary significantly from a Poisson distribution ($p > 0.01$) (5).
- The data must be acceptable by the test of adequacy ($p > 0.01$) (5).
- The test for mutagenicity (i.e., the slope of the curve) must be significant ($p < 0.01$) (5).
- At least a twofold increase must have occurred over spontaneous levels at one or more doses.
- A dose-response curve with some type of "regular curve" must have been seen (e.g., a single high response would not be acceptable).
- All controls must have given expected responses.
- Histidine cross-feeding and/or contamination must not have been shown to occur.

Data fitting all of the above criteria are clearly positive. However, a positive response may occur in which all of the above criteria do not apply. The following are the criteria used as guidelines by the author in these cases:

- In all cases the controls give expected responses and the histidine cross-feeding and/or contamination is never shown to occur.
- When the data depart significantly from a Poisson distribution ($p < 0.01$), but all other items hold, and a single dose or plate causes deviation from the Poisson distribution, the response can be considered positive.
- When the test for adequacy shows a significant departure from the expected response ($p < 0.01$), but all other items hold, and a single dose or plate causes this significance, the response can be considered positive.
- If no dose gives a twofold increase over spontaneous but all other items hold, the response can be considered a borderline positive.

In each of these cases quantitative comparisons are questionable and, if possible, the tests should be repeated.

Since most positive results are easily distinguished, experiments are designed to give some definition to a negative result. The following are the minimal testing requirements used to define a negative test response:

- A minimum of 5 doses at half-log intervals with the highest dose being highly toxic or presented at a minimum of 5 mg per plate
- Spontaneous and positive controls done at least in duplicate and providing the expected response
- Positive controls (in duplicate) for any bacterial/microsomal activation combination used are within normal ranges.
- At least two replicates per dose

- At least one replicate experiment, if possible, done with a narrower dose range
- Tested in TA1535, TA1537, TA98, and TA100
- A negative response with the sterility controls
- Restreaked colonies grew on histidine-free media.
- Lack of any indication of mutagenicity (e.g., With the Stead model (5), the test for mutagenicity is not significant [$p > 0.01$]).

A variety of ways to present summarized data is available. deSerres and Shelby, however, suggest that summarized data not be presented without supporting data (6). The following is a listing of some of the ways to summarize and compare data:

- Fold increase or mutagenic index: Average plate counts divided by average spontaneous count for the same bacterial strain
- Net increase in revertants: Average counts or individual counts minus average spontaneous count. (Generally considered unacceptable)
- Specific activity: Slope of the linear portion of the dose-response curve
- Specific response activity: Calculated dose needed to give a specific number of revertants per plate (e.g., 200 rev/plate) for a specific strain
- Maximum response: Maximum number of revertants per plate seen
- Model slope: Revertants/plate/dose as calculated from a curve that models the test response

These methods are used to compare the results of a series of tests, but do not necessarily reveal the adequacy or positiveness of the results. Several methods for examining *Salmonella* plate test data have been or are being published. The model by Stead et al. supplies the most stringent criteria for acceptance of data (5).

PART II

HEALTH EFFECTS RESEARCH LABORATORY STEPWISE EXPERIMENTAL PROTOCOLS

SECTION 1

INITIAL EQUIPMENT LIST

PERMANENT MAJOR EQUIPMENT

Delineated below is a list of equipment essential for successful completion of biological bioassays and which must be maintained at all times to assure accurate test results:

- Biological safety cabinet (hood)
- Autoclave
- Incubators
- Refrigerator(s) and freezers
- Balances (both an analytical and a top-loader balance)
- Bacterial colony counter (any standard model)
- Water baths (for media preparation and maintaining overlay tubes)
- Shaker heater or water bath (for culturing media and for preincubation test)

The biological safety cabinet (hood) must be designed to protect both personnel and the work area. The hood's intake air must be high-efficiency, HEPA-filtered, and have no greater than 30% recirculation. Airflow over the work surface is to be directed away from personnel and exhausted. All exhaust air should flow through a trap appropriate for removing organic materials.

The autoclave chosen must be of good quality and be able to maintain standard autoclave conditions. Since the autoclave is used to house large reagent bottles and flasks, size is also a necessary consideration.

Forced air incubators are preferable to other available types because they allow better temperature control. All incubators used must maintain a uniform temperature (37°C ±.5°C) throughout the chamber. Air from the incubators should be directly or indirectly exhausted.

Refrigerators and freezers must be lockable. Any refrigerators used for strong solvents or organic materials must be explosion-proof. Freezer temperature for bacteria storage is -80°C.

OPTIONAL MAJOR EQUIPMENT

To increase the accuracy of test results, other major equipment may be needed, including:

- Automatic colony counter
- Automatic plate pourer
- Dri-block heaters for 13 x 100 mm test tubes

The range of reliability and any needed correction curves must be established for the automatic colony counter. The counter must also be checked routinely for accuracy. The automatic plate pourer must be able to fill plates with a precise amount of media under sterile conditions. Dri-block heaters for 13 x 100-mm test tubes may be used in place of a water bath for overlay tubes.

MINOR EQUIPMENT

The list below contains minor equipment necessary for completion of *Salmonella* tests:

- Automatic pipettes
- Glassware
- Plastic bag sealer
- Automatic repipette

Separate pipettes are needed for the various volumes used in the *Salmonella* test. Glassware used should include Erlenmeyer flasks (4-L, 2-L, 500-mL and 50-mL), reagent bottles (1-L and 200-mL), and graduated cylinders (1-L, 100-mL, and 50-mL). An automatic repipette is useful for S-9 and bacterial broth. The repipette must be calibrated and routinely checked.

DISPOSABLE EQUIPMENT

All of the following equipment is disposable:

- Automatic pipette tips
- Plastic bags for incubation of plates
- Hazardous waste bags
- Latex gloves
- Petri dishes (15 x 100-mm, sterile)
- Test tubes (13 x 100-mm)
- Test tube caps for 13 x 100-mm tubes

- Pipettes (TD, disposable, glass; 1-mL, 2-mL, 5-mL, and 10-mL)
- Personnel gowns
- Table top covers

SECTION 2

MEDIA PREPARATION

50X VBME STOCK SALTS SOLUTION

To prepare 50X VBME stock salts solution, measure out 600 mL deionized water into a clean 2-L Erlenmeyer flask. Add magnet stirring bar and heat on a hot plate, with stirring. After water is moderately hot (about 45°C), add the following chemicals slowly in the following order:

(1)	Magnesium sulfate ($\mathrm{MgSO_4 \cdot 7H_2O}$, MW = 246.5)	10 g
(2)	Citric acid monohydrate ($\mathrm{HOCCOOH\,[CH_2COOH_2]_2 \cdot H_2O}$, MW = 210.14)	100 g
(3)	Potassium phosphate dibasic anhydrous ($\mathrm{K_2HPO_4}$, MW = 174.18)	500 g
(4)	Sodium ammonium phosphate ($\mathrm{Na[NH_4]HPO_4 \cdot 4H_2O}$, MW = 209.07)	175 g

After all chemicals are dissolved, pour the solution into a glass 1-L graduated cylinder and bring to 1000 mL volume by adding deionized water. Then filter the solution through a Buchner funnel (with Whatman #1 filter paper) into a vacuum flask. After filtering is complete, pour 500 mL of the solution into each of two clean 1-L Wheaton bottles labeled with 50X VBME solution and date prepared. Autoclave the two bottles, loosely capped, for 25 min on slow exhaust. After the bottles and their contents are well cooled, tighten the caps and place the bottles in cabinet. Do not store for more than two months.

HISTIDINE/BIOTIN SOLUTION

To prepare the histidine/biotin solution, first measure out 1000 mL deionized water and pour into a clean 2-L Erlenmeyer flask. Cover the flask with tinfoil, then autoclave for 30 min on slow exhaust. After the water in the flask cools slightly, add 0.0525 g l-histidine and 0.0976 g biotin and shake vigorously to dissolve.

Turn hood on and allow to run for a few minutes. Pour the His/Bio solution into a sterile 1000-mL Wheaton bottle labeled with "His/Bio solution, sterile." Also label the bottle with the date prepared. Store in refrigerator for no longer than one week.

BIOTIN SOLUTION

The biotin solution should be prepared as the above solution except the biotin solution uses only 0.0976 g biotin and no histidine. Label as Bio solution.

PREPARATION OF VBME PLATES (APPROXIMATELY 100 PLATES)

Materials needed to prepare 3 L of media are:

- One 4-L Erlenmeyer flask
- One 500-mL Erlenmeyer flask
- Difco agar
- Dextrose
- His/Bio solution
- 50X VBME solution
- One sterile glass 100-mL graduated cylinder
- One sterile glass 50-mL graduated cylinder

Into a 4-L flask add 2655 mL of deionized water and 45 g of Difco agar. Cover the flask with tinfoil and shake gently. Then place the flask on a hot plate and heat, with stirring, until agar dissolves. Watch carefully to avoid agar burning to the bottom of the flask.

Pour 200 mL of deionized water into a 500-mL flask. After adding 60 g of dextrose, cover with tinfoil and shake gently. Place in a 55°C water bath to dissolve dextrose. After agar and dextrose are dissolved, autoclave both flasks for 35 min using slow exhaust. After flasks cool slightly, add dextrose solution to agar.

Place the 4-L flask in a 55°C water bath. After 15 min, measure out 60 mL of 50X VBME solution using a sterile 100-mL graduated cylinder and pour into the flask. Mix and return to water bath. After an additional 15-min wait, add 30 mL of the His/Bio solution using a sterile graduated cylinder into the flask. Mix and return to the 55°C water bath. Using an automatic plate pourer, dispense 30 mL of the media into each 100 x 15-mm petri plate. After agar solidifies, shake off condensation remaining on the lids of the plates.

Incubate the plates (inverted) at 37°C overnight. After examining them for contamination, bag the plates (20 plates per bag). Write the batch number and date on each bag and record the total number of plates made.

PREPARATION OF OTHER NECESSARY SOLUTIONS

Nutrient Broth

Dissolve the ingredients listed below and dispense into desired vessels. Then autoclave.

• Difco bacto nutrient broth	8.0 g
• Sodium chloride	5.0 g
• Distilled water	1.0 L

Nutrient Agar

Mix the ingredients below and autoclave for 15 min. Swirl melted agar to mix and then pour onto plates.

• Difco bacto nutrient broth	8.0 g
• Sodium chloride	5.0 g
• Agar	15.0 g
• Distilled water	1.0 L

Top Agar

Dissolve 6 g bacto-agar and 5 g NaCl completely in 1000 mL deionized water and dispense the mixture into clean screw-capped bottles (100 mL/bottle) or culture tubes (2.1 mL/tube). Autoclave for 15 min at 121°C. Use slow exhaust. Before use, melt in autoclave or microwave and cool to 45°C.

$MgCl_2$ - KCl Solution

Autoclave a solution of 81.32 g $MgCL_2 \cdot 6H_2O$, 123.02 g KCl, and 1000 mL deionized water for 20 min.

Sodium Phosphate Buffer (0.2 M)

Mix and autoclave 7.76 g KH_2PO_4, 20.30 g Na_2HPO_4, and 1000 mL deionized water for 20 min. The pH should be adjusted to 7.4 with sodium hydroxide.

Glucose-6-Phosphate (G-6-P)

Immediately before the experiment, 1.41 mg G-6-P per mL of S-9 mix is added to sterile deionized water to obtain the needed amounts of G-6-P.

Nicotinamide Adenine Dinucleotide Phosphate (NADP)

Immediately before the experiment, 3.06 mg NADP per mL of S-9 mix is added to sterile deionized water to create the needed amounts of NADP. Filter and sterilize the solution before use.

SECTION 3

PREPARATION OF RAT LIVER S-9 ACTIVATION MIXTURE

COMPONENTS OF S-9 MIXTURE

The five components of the rat liver S-9 activation mixture are:

- Salt solution ($MgCl \cdot 6H_2O$ - 0.4 M; KCl - 1.65 M)
- G-6-P (1.41 mg/mL S-9 mix)
- NADP (3.06 mg/mL S-9 mix)
- S-9 (usually between 0.05 and 0.1 mL S-9/mL S-9 mix)
- Ames buffer (KH_2PO_4 - 7.76 g/L; Na_2HPO_4 - 20.3 g/L), pH = 7.4

To dissolve G-6-P and NADP, add sterile deionized water. The volume of water is determined by summing the amount of salt solution, buffer, and S-9 added and subtracting this volume from the total amount of S-9 mix being prepared. Sterile filter the NADP solution through a 0.45-μm filter.

The amount of S-9 used in the S-9 mixture depends upon the activity of each new lot of S-9. But, as stated above, the amount is usually between 0.05 and 0.1 mL S-9/mL S-9 mix.

Buffer always makes up one-half of the S-9 mix.

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PROTOCOL FOR PREPARATION OF RAT LIVER S-9 FRACTION

Five days prior to sacrifice, inoculate rats weighing approximately 200 g intraperitoneally with Aroclor 1254 in corn oil (200 mg/mL). The delivered dose is 500 mg Aroclor 1254/kg of body weight. One day prior to sacrifice, take the animals off food, but provide water *ad libitum*. Sacrifice animals by cervical dislocation 5 days after injection. The following procedures are performed in a vertical laminar flow hood, employing aseptic techniques:

- Remove the livers, mince several times, then transfer to a preweighed beaker on ice containing sterile, cold 1.15% KCl.
- Following removal of all the livers (less than 45 min), reweigh the beakers and record the weight of the livers.
- Rinse the liver fragments several times with sterile 1.15% KCl and further mince until tissue fragments of approximately 2-3 mm^2 are obtained. Rinse the liver mince again several times with sterile 1.15% KCl.
- Following the last KCl rinse, transfer the minced liver to a beaker containing sterile 0.25 M sucrose (on ice). Then rinse the minced liver twice, decanting as much of the last sucrose rinse as possible without losing the liver.
- Add sterile 0.25 M sucrose at a 2:1 ratio (sucrose volume:liver weight).
- Homogenize the minced tissue using a 50-mL Potter-Elvehjen homogenizer and pool. One complete downward and upward stroke is used for homogenization.
- Centrifuge the homogenate at 9000 x g for 25 min at 4°C.
- Decant the supernatant through sterile gauze held on ice. Then aliquot a 2 mL volume of S-9 into individual 3 mL polypropylene freezing vials, and immediately quick freeze using liquid nitrogen. Store at -80°C in a Revco low-temperature freezer.

- Randomly select several vials for quality assurance and protein determinations.

- Record observations on computerized information sheets.

SECTION 4

SALMONELLA TESTER STRAINS

OVERVIEW

Presently there are five standard mutant strains of *Salmonella typhimurium* used for routine testing. These five strains -- TA98, TA100, TA1535, TA1537, and TA1538 -- are obtained by HERL from Dr. Bruce Ames at the University of California, Berkeley. Other specialized strains are described as used for each additional test. The sensitivity of all five tester strains has been enhanced by the deep rough (*rfa*) and *uvr* B mutations. The *rfa* mutation eliminates the polysaccharide side chain of the lipopolysaccharide layer coating the bacterial surface making the bacteria cell wall more permeable to higher molecular weight chemicals. The deletion of the *uvr* B gene causes a loss of the cell's excision repair capabilities. The *uvr* B detection also includes deletion of the biotin (*bio*) gene; therefore, all five tester strains also require biotin as well as histidine. Two of the strains, TA98 and TA100, also contain R-factor plasmids which provide ampicillin resistance. These two strains were developed by transferring plasmids into strains TA1538 and TA1535, respectively. This plasmid seems to increase the sensitivity of these two strains by causing error-prone recombination repair; however, the exact mechanism by which the R-factor enhances mutagenesis is not known. The TA1535 and TA100 strains can

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detect mutagens which cause base-pair substitutions. The other strains, TA1537, TA1538, and TA98, are capable of detecting various kinds of frameshift mutagens. The TA100 strain is also sensitive to many frameshift mutagens. Dr. Herbert Rosenkranz has developed a series of strains that are nitroreductase-deficient (7). These and other strains are also available and are listed in Table 2-1.

TABLE 2-1. CHARACTERISTICS OF *SALMONELLA TYPHIMURIUM* TESTER STRAINS

	Additional Mutations					
Strain	*his*-Mutation	LPS	Repair	R	ND	Spontaneous reversions/plate
			Common Tester Strains			
TA98	D3052	rfa	Δuvr B	Yes	No	15-75
TA100	G46	rfa	Δuvr B	Yes	No	80-200
TA1535	G46	rfa	Δuvr B	No	No	5-50
TA1537	C3076	rfa	Δuvr B	No	No	2-25
TA1538	D3052	rfa	Δuvr B	No	No	5-40
			Additional Tester Strains			
TA98-FRI	D3052	rfa	Δuvr B	Yes	Yes	
TA100-FRI	G46	rfa	Δuvr B	Yes	Yes	
TA98/NR	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/NR/1, 8 DNP$_1$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/NR/1, 8 DNP$_2$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/NR/1, 8 DNP$_5$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_1$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_8$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_{11}$	D3052	rfa	Δuvr B	Yes	Yes	
TA98/1, 8 DNP$_6$/NR$_{12}$	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 301	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 302	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 303	D3052	rfa	Δuvr B	Yes	Yes	
TA98 NIR 304	D3052	rfa	Δuvr B	Yes	Yes	

(continued)

TABLE 2-1. (continued)

	Additional Mutations					
Strain	*his*-Mutation	LPS	Repair	R	ND	Spontaneous reversions/plate
			Additional Tester Strains			
TA100/NR	G46	rfa	Δuvr B	Yes	Yes	
98ND	G46	rfa	Δuvr B	Yes	Yes	
100ND	G46	rfa	Δuvr B	Yes	Yes	
TA1978	D3052	rfa	+	No	No	
TA1977	C3076	rfa	+	No	No	
TA1975	G46	rfa	+	No	No	
TA1964	D3052	Δgal	Δuvr B	No	No	
TA1532	C3076	Δgal	Δuvr B	No	No	
TA1530	G46	Δgal	Δuvr B	No	No	
TA2641	D3052	Δgal	Δuvr B	Yes	No	
TA2631	G46	Δgal	Δuvr B	Yes	No	
TA2637	C3076	rfa	Δuvr B	Yes	No	
TA1534	D3052	+	Δuvr B	No	No	
TA1952	C3076	+	Δuvr B	No	No	
TA1950	G46	+	Δuvr B	No	No	
TA2410	G46	+	Δuvr B	Yes	No	
TM35	+	rfa	Δuvr B	No	No	
TM677	+	rfa	Δuvr B	Yes	No	
			Parental Strains			
LT2	+	+	+	No	No	
G46	G46	+	+	No	No	
C3076	C3076	+	+	No	No	
D3052	D3052	+	+	No	No	

ROUTINE CHECK OF STRAINS

Each strain should be checked for five specific characteristics on a routine basis. These checks are essential, especially in the case of the unstable plasmid-containing strains TA100 and TA98. Characterization of the tester strains should be performed each time the strains are grown from frozen permanents, according to the procedure discussed below.

Histidine Requirement

Add a nutrient broth culture (0.1 mL) of each strain to an overlay agar tube and pour onto a minimal media plate both with and without histidine (0.1 mL of 0.1 M l-histidine) and biotin (0.1 mL of 0.5 mM biotin). After 72 h at 37°C, growth should be observed on the plates with histidine and biotin. No growth should appear on the non-supplemented plates; however, spontaneous revertant colonies from the overnight broth culture will appear upon this non-supplemented media.

Spontaneous Reversion

The spontaneous reversion rate should be within the range recorded in Table 2-1. A time course record of the results should be kept. The method is as follows:

- Melt top agar in autoclave or microwave oven. Pipet 2.0 mL into each sterile, capped, 13 x 100-mm culture tube. The culture tubes should be preheated and maintained at 45°C in a water bath or heating block.
- For each tester strain, add 0.1 mL of broth culture to a culture tube, mix, and pour onto the surface of a minimal agar plate. Spread the top agar uniformly over the surface of the minimal agar by gently rotating the plate. Repeat this process three times for each strain.
- An optional procedure is to repeat the second step with the addition of 0.5 mL of the S-9 mix being added to overlay before pouring.

- Place the petri dishes on a flat dry surface and allow overlay to harden. Incubate at 37°C for 72 h in an inverted position.

- Examine the background lawn of bacteria visually for proper growth. Examining the background lawn with a microscope is sometimes useful.

- Count the number of spontaneous revertant colonies and record the number. Determine whether or not the average number of colonies per plate is within an acceptable range.

Deep Rough (rfa) Character

Pipet 0.1 mL of an overnight bacterial growth (approximately 10^8 bacteria) into a 2.0-mL top agar overlay. Pour and spread the resultant mixture onto a nutrient agar plate. With sterile forceps, place a filter paper disc containing 10 µL of a 1-mg/mL solution of crystal violet in the center of the plate. Incubate for 18 to 24 h at 37°C. All tester strains should indicate the presence of the rfa mutation by a circular zone of growth inhibition (approximately 14 mm in diameter) around the disc.

Ampicillin-Resistant R-Factor Plasmid

Pipet 0.02 mL (20 µL) of an overnight broth culture (approximately 2×10^7 bacteria) onto a 2-mL top agar overlay. Pour and spread this mixture onto a nutrient agar plate. With sterile forceps, place a 10-µg ampicillin disc in the center of the plate. Incubate for 18 to 24 h at 37°C. Strains containing the R-factor plasmid (such as TA98 and TA100) are not inhibited by ampicillin; whereas, all other strains demonstrate a zone of growth inhibition (approximately 20-24 mm in diameter) around the disc.

Excision Repair System (uvr B)

Pipet 0.05 mL (50 μL) of an overnight broth culture (approximately 5×10^7 bacteria) onto a nutrient agar plate. Using bent glass tubing (a "hockey stick"), spread the bacteria over the agar surface. Irradiate half of the plate at 58 cm under the germicidal lamp within a Bioguard hood for 10 s for strains not carrying the R-factor plasmid and for 20 s for strains with the R-factor plasmid.

An optional procedure is to irradiate half of the plate under a 15-watt germicidal lamp at 33 cm for 8 s. In both procedures, incubate for 18 to 24 h at 37°C. Growth should occur only on the unirradiated portion of the plate.

USE OF MASTER PLATES

To avoid continued reuse of permanent stocks and/or the creation of large numbers of frozen aliquots, master plates may be prepared and used for 1 month. The following method is suggested:

- Thaw frozen broth culture. Using a needle and syringe, add 1 mL of the broth culture to 20 mL of the nutrient broth and incubate with constant shaking (80-100 rpm) at 37°C for 16 h. Inoculate the nutrient agar master plates with 0.05 mL of the broth culture.
- Master plates will be nutrient agar plates.
- Streak each plate with the appropriate tester strain. Label plates appropriately.
- Incubate plates at 37°C for 48 h.
- Use the remaining broth cultures to perform tester strain function.

- After 48 h, store the plates at 4°C in the dark. It is advantageous to seal the plates with tape in order to prevent drying and/or to facilitate streaking from the master plates within a cold room.

- When tests are performed, a single isolated colony should be used to inoculate the overnight broth culture. This prevents a mixed population growth.

SECTION 5

THE PLATE INCORPORATION TEST

DESIGN OF THE EXPERIMENT

Each routine test must consider the following parameters: (1) The amount of sample available for test, (2) the number of tester strains to be used, (3) performance of the test with and/or without exogenous activation, (4) spontaneous and positive controls, (5) the doses of the sample to be used, and (6) the number of replicate plates per dose. Table 2-2 provides some examples of testing routines with an estimation of the sample amount and number of plates needed.

Although complete testing cannot be done without the use of each indicator strain both with and without activation, sample size may limit the amount of testing possible. By setting a priority on the strains to be used, one can increase the likelihood of detecting active compounds. Priority assigned to the strains depends upon the type of chemical or substance to be tested. Structure-activity and source-activity relationships can be used to guide the researcher. In general, these sample size constraints and other constraints determine the number of strains to be used.

TABLE 2-2. PLANNING SCHEME FOR SALMONELLA PLATE INCORPORATION TEST

Dose (µL/plate) per sample											
500		3	2	1	1	-	-	-	-	-	-
300		3	2	1	3	3	2	1	1	-	-
100		3	2	1	3	3	2	1	3	3	3
50		3	2	1	3	3	2	1	3	3	3
30		3	2	1	3	3	2	1	3	3	3
10		3	2	1	3	3	2	1	3	3	3
		mL (# plates)									
+ OR-MA	1 strain:	3.0 (18)	2.0 (12)	1.0 (16)	2.0 (16)	1.5 (15)	1.0 (10)	0.5 (5)	0.9 (13)	0.6 (12)	0.4 (8)
	2 strains:	6.0 (36)	4.0 (24)	2.0 (12)	4.0 (32)	3.0 (30)	2.0 (20)	1.0 (10)	1.8 (26)	1.2 (24)	0.8 (16)
	3 strains:	9.0 (54)	6.0 (36)	3.0 (18)	6.0 (48)	4.5 (45)	3.0 (30)	1.5 (15)	2.7 (39)	1.8 (36)	1.2 (24)
	4 strains:	12.0 (72)	8.0 (48)	4.0 (24)	8.0 (64)	6.0 (60)	4.0 (40)	2.0 (20)	3.6 (52)	2.4 (48)	1.6 (32)
	5 strains:	15.0 (90)	10.0 (60)	5.0 (30)	10.0 (80)	7.5 (75)	5.0 (50)	2.5 (25)	4.5 (68)	3.0 (60)	2.0 (40)
+ AND-MA	1 strain:	6.0 (36)	4.0 (24)	2.0 (12)	4.0 (32)	3.0 (30)	2.0 (20)	1.0 (10)	1.8 (26)	1.2 (24)	0.8 (16)
	2 strains:	12.0 (72)	8.0 (48)	4.0 (24)	8.0 (64)	6.0 (60)	4.0 (40)	2.0 (20)	3.6 (52)	2.4 (48)	1.6 (32)
	3 strains:	18.0 (108)	12.0 (72)	6.0 (36)	12.0 (96)	9.0 (90)	6.0 (60)	3.0 (30)	5.7 (78)	3.6 (72)	2.4 (48)
	4 strains:	24.0 (144)	16.0 (96)	8.0 (48)	16.0 (128)	12.0 (120)	8.0 (80)	4.0 (40)	7.2 (104)	4.8 (96)	3.2 (64)
	5 strains:	30.0 (180)	20.0 (120)	10.0 (60)	20.0 (160)	15.0 (150)	10.0 (100)	5.0 (50)	9.0 (130)	6.0 (120)	4.0 (80)

(# Samples) X (indicated # plates for selected scheme) + (9) X (# strains) = number plates needed.
Volume of each sample needed given directly in table.
(Volume) X (concentration/volume) = amount of sample needed.

Although exogenous activation is difficult to define, routine testing should be done both with and without a mammalian activation system.

The range of doses in which the dose-response exists consists of nearly six orders of magnitude; however, the dose range in which a substance shows a dose-responsive curve may be very narrow, especially for complex mixtures. The minimum requirements within the HERL laboratory are five doses at half-log intervals. The highest dose should demonstrate toxicity, and the lower doses should be in the spontaneous range. For application of statistical methods, the laboratory expects three plates per dose and three doses when possible.

PROTOCOL

Select one well-grown colony from the master plate for each strain to be used and inoculate properly labeled nutrient broth flasks (20 mL per flask). Incubate these flasks with constant agitation for 16 h at 37°C.

On the day of the experiment, melt 2 mL of the top agar within the 13 x 100-mm tubes, and cool the tubes to 45°C in a water bath. It is also helpful to warm plates to 37°C prior to use, or to incubate plates overnight at 37°C if the contamination of plates is a problem.

Controls

Prepare spontaneous control plates adding 0.1 mL of bacterial broth culture and a selected amount of solvent used for test substance to the overlay tube. Gently vortex, pour the mixture onto the plate, and spread it evenly across the plate.

For positive controls without activation, add 0.1 mL of bacterial broth culture and 100 µL of a selected concentration of control chemical in appropriate solvent to the overlay tube. Gently vortex, pour onto plate, and spread evenly across the plate. When possible, use the same solvent as is used with the test substance.

For positive controls with activation, add 0.1 mL of bacterial broth culture, 0.5 mL of the activation mixture, 100 µL of the selected concentration of control chemical in the appropriate solvent to the overlay tube. Gently vortex, pour onto plate, and spread evenly across the plate. When possible, use the same solvent as is used with the test substance.

The Test Substance

Add 0.1 mL of the bacterial broth culture. Next add the appropriate concentration of test substance. When activation is to be used, also add 0.5 mL of the activation mixture to the overlay tube. Gently vortex, pour the mixture onto the plate, and spread it evenly.

After overlays have hardened, invert and incubate in the dark at 37°C for 72 h.

After this 72-h period, examine each plate for background growth. Then count the number of colonies per plate. If there are fewer than 20 colonies per plate, counting can be done visually; otherwise, electronic counting machines may be used.

Select at random from test substance plates colonies that are restreaked onto minimal media. Check the activation mixture and test substances by overlaying them onto VBME agar plates.

Record all results onto computer-compatible sheets; the responsible investigator should initial all sheets.

SECTION 6

THE SPOT TEST

OVERVIEW AND EXPERIMENTAL DESIGN

The spot test is a simple test used to determine qualitatively whether or not a substance is mutagenic. Because of the amount of test substance used and the limited area into which the sample is placed, the spot test is less sensitive than the plate test. The spot test is also more likely to provide false negatives because of a lack of responsiveness to narrow dose ranges and a lack of diffusion of some substances. The test can be used, however, to obtain preliminary indications of mutagenicity and to obtain an estimate of active dose levels.

In this simple test, differing concentrations of the sample are generally placed in the center of the selective media plates. Each plate is then examined for zones of killing and increased mutant colonies.

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PROTOCOL

Select one well-grown colony from the master plate for each strain to be used and inoculate properly labeled nutrient broth flask. Incubate flasks for 16 h at 37°C with constant agitation.

On the day of the experiment, melt the top agar contained in the 13 x 100-mm sterile tubes and cool to 45°C in a water bath.

For each plate, add 0.1 mL of the overnight bacterial broth. If the S-9 is to be used, add 0.5 mL of the S-9 mix. After vortexing gently, pour the mixture onto the plate and spread evenly.

When the agar has hardened (approximately 15 min), add a sterile disc to the center of the plate and add the appropriate concentration of the sample to the disc. Also complete appropriate positive controls.

Incubate all petri dishes inverted at 37°C for 2 days. After incubation, examine all plates for zones of killing and mutagenicity. Then record results in a permanent notebook and on computerized results sheets.

SECTION 7

FORWARD MUTATION TO 8-AZAGUANINE RESISTANCE IN *SALMONELLA TYPHIMURIUM*

STRAINS (TM35 AND TM677)

Strain TM35 is a spontaneous H^+ revertant of Ames TA1535. It is mated with TA2000 to yield the ampicillin-resistant strain TM677. Strains TM35 and TM677 are obtained from Thomas R. Skopek at the Massachusetts Institute of Technology, Cambridge, Massachusetts.

MEDIA

To prepare the Vogel-Bonner minimal E medium 50X stock solution, add the chemicals listed below in the order given to 600 mL of deionized water. Then filter the solution through Whatman No. 1 filter paper and autoclave for 25 min at 121°C.

(1)	Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	10 g
(2)	Citric acid monohydrate ($HOCCOOH\ [CH_2COOH_2]_2 \cdot H_2O$)	100 g
(3)	Potassium phosphate dibasic anhydrous (K_2HPO_4)	500 g

(4) Sodium ammonium phosphate ($Na[NH_4]HPO_4 \cdot 4H_2O$)	175 g

Other media used for the test include:

- Dulbecco's - Phosphate buffered saline (PBS)
- Nutrient broth (Difco)
- Biotin - 50X stock solution (0.61 mg/mL)
- Glucose - 50X stock solution (0.5 g/mL)
- 8-Azaguanine (Sigma) (16.25 mg/mL stock solution)
- Vogel-Bonner minimal E medium agar plates - 30 mL agar/plate

FORWARD MUTATION ASSAY PROCEDURE

Inoculate a 20-mL nutrient broth with a single colony from a master plate. Incubate this new culture 16 h in a shaking incubator at 37°C. Then using a refrigerated Sorvall centrifuge, spin the culture down at 3000 rpm for 15 min. Pour the supernatant off and resuspend the button in enough PBS to obtain a bacterial concentration of 5×10^8 bacteria/mL.

To prepare the minimal Eagle's medium reaction mixture, dissolve 1 mL VBME salt solution, 1 mL biotin solution, 2 mL glucose solution, and 4 mL of the 5×10^8 bacteria (either TM35 or TM677)/mL PBS suspension in 42 mL sterile deionized water. Incubate this mixture for 1 h at 37°C in a shaking incubator. This incubation period restores the bacteria to the log phase of growth.

After incubation, pipet 4.9 mL of the reaction mixture into 30-mL centrifuge tubes. Deliver the compound to be tested in 100 μL of DMSO to make a total volume of 5 mL. Use MNNG at a concentration of 0.3 μg/mL as a positive control for samples not requiring activation. For samples requiring activation, use 2-Anthramine at a concentration of 20 μg/mL as a control. A spontaneous control consisting of 4.9 mL of the reaction mixture and 100 μL of DMSO can also be used. Incubate control and test sample tubes for 2 h in a shaking incubator at 37°C.

For compounds requiring activation, pipet 4.0 mL of the reaction mixture into 30-mL centrifuge tubes containing a mixture of 0.5 mL S-9, 0.4 mL of NADP and G-6-P solution, 0.1 mL of microsome salt solution, and 0.1 mL of the compound to be tested.

NADP should be at a concentration of 3.06 mg/mL and G-6-P at a concentration of 1.41 mg/mL in the centrifuge tubes. The microsome salt solution used is identical to the one used in the Ames test. The S-9 mixture, prepared from rats induced with Aroclor 1254, should be made fresh each month and stored at -80°C. Also, the researcher should include a spontaneous control by substituting 100 μL of DMSO instead of the test compound. Incubate the 30-mL tubes for 2 h at 37°C in a non-shaking incubator.

After incubation, spin all tubes down at 3000 rpm for 15 min in a refrigerated Sorval centrifuge. Pour the supernatant off and resuspend the bacteria in 5 mL of PBS.

Plate the mutants by pipetting 100 μL of the resuspended bacteria and 100 μL of the 8-azaguanine stock solution into 2.5 mL of top agar and pour onto

VBME agar plates (3 plates per tube). After 48 h, count the mutant colonies with an Artek colony counter.

Make a 10^4 bacteria dilution using 100 μL from each tube. Then pipet 100 μL of the 10^4 dilution into 2.5 mL of top agar and plate on VBME plates (3 plates per tube). Incubate the plates for 48 h at 37°C and count surviving colonies with an Artek colony counter.

The 8-azaguanine concentration in the VBME plates should be 50 μg/mL of agar. Calculate the fraction of 8-azaguanine-resistant bacteria as follows:

$$\frac{\text{Average \# mutants on 8-Ag plates}}{\text{Average \# survivors on VBME plates without 8-Ag}} \Big/ 10^4 \text{ bacteria}$$

SECTION 8

FLUCTUATION TEST (WITH SALMONELLA AND NO METABOLIC ACTIVATION)

Instructions for this test and detailed information are available in the literature (8, 9).

SOLUTIONS

Solutions necessary for this test require the following ingredients:

- DM (Davis-Mingioli) salt
- K_2HPO_4 (7 g)
- KH_2PO_4 (2 g)
- $(NH_4)_2SO_4$ (1 g)
- Trisodium citrate (0.25 g)
- $MgSO_4$ (0.1 g)
- Deionized water (1000 mL; autoclaved)

Each of these ingredient compounds should be autoclaved and refrigerated before use.

Solution A

To prepare, dissolve 0.61 g $MgCl_2 \cdot 6H_2O$ in 60 mL water. Adjust the pH to 7.5 by adding 1N HCl (~ 20 mL), then add water to a total volume of 100 mL.

Autoclave the solution and adjust the pH. Refrigerate the solution to preserve for future use.

Solution B

Add water to 21.2 g of 0.62 M sucrose until the solution reaches a volume of approximately 70 mL. Autoclave the resultant solution and add sterile water to a final volume of 100 mL.

Solution C

Mix 20 mL of Solution A, 20 mL of Solution B, and 10 mL of sterile deionized water.

DM Salts with Histidine, Biotin and Glucose (DM^{+++})

To prepare a DM salts solution with histidine, biotin, and glucose (DM^{+++}), mix 100 mL of DM salts, 1.5 mg l-histidine, 0.8 mg biotin, and 0.8 g glucose.

Stock Bromcresol Purple

For this solution, mix 5 mg bromcresol purple (BCP) per 1 mL of DM salts. (Note: Use 0.1 mL/100 mL DM salts for 5 μg/mL.)

PBS Dulbecco-A Phosphate Buffered Saline

This premixed solution should be autoclaved before use.

PROCEDURE

On the day before the test, inoculate three 20-mL flasks of nutrient broth with one colony each of the Salmonella strain to be used. Incubate at 37°C in shaking incubator for 16 h.

On the morning of the test, combine the three flasks of Salmonella into two centrifuge tubes. Spin at 2000 rpm for 20 min in a Sorvall RC2B centrifuge. Resuspend the pellets in sterile PBS at a total volume of 5 mL. Then dilute an aliquot 1:4 with PBS to an OD of 420 nm (~ 4 x 10^9 bacteria/mL).

On the afternoon of the test, prepare "pot" of buffer:

- 10 mL DM^{+++}
- 10 mL Solution C
- 30 mL DM salts

50 mL buffer

Add a sufficient volume of Salmonella culture to yield approximately 0.04 x 10^7 bacteria/0.1 mL to be plated. Aliquot "pot" into 10 tubes at 5 mL/tube. Then add compound(s) to be tested. Dispense each tube into a Linbro (Linbro Scientific, Inc., 143 Leeder Hill Drive, P.O. Box 6187, Hamden, CT 06517) tissue culture multi-dish at 0.1 mL/well (24 wells/dish).

Dilute an 0.1-mL sample with each tube 10^6 with sterile PBS. Plate 0.5 mL of each dilution in 2 mL of top agar on nutrient agar plates (triplicate plates/tube). Incubate at 37°C for 72 h before counting colonies to determine the number of bacteria initially added to wells.

Incubate the Linbro dishes at 37°C overnight (~ 19 h). Then prepare a solution of bromcresol purple in DM salts (5 μg/mL) containing 0.4% glucose. Aseptically add 2 mL of DM salts + glucose + BCP to each well of each Linbro dish.

Remove the contents of 1 well/dish to a sterile culture tube. Plate 0.5 mL in 2 mL of top agar on a VBME plate (triplicate plates/tube) to determine mutants. Dilute 10^6 with sterile PBS and plate 0.5 mL of the dilution in 2 mL top agar on nutrient agar plates (in triplicate) to determine number of bacteria in wells. Incubate plates at 37°C for 72 h before counting colonies.

Return the Linbro dishes to the incubator at 37°C for 72 h. Count the number of wells which have changed from purple to yellow. Record as the number of purple wells + yellow wells/total number of wells used in a dish, i.e., 23. If the color change is questionable, count the well as a negative (purple). Plot the number of positive wells versus the concentration of the compound/well or the total concentration in dish (concentration/well x 24 wells).

To test for phenocopy colonies, restreak randomly-selected colonies from several of the VBME plates onto minimal media to determine if growth occurs without histidine.

SECTION 9

RECORDING OF RESULTS AND QUALITY CONTROL

Record all results in a permanent laboratory record book and on standard forms for computerization (see Part III). Five forms are currently available for recording data for keypunching. The "Sample Identification Form" allows the user to register an identification code and an associated description of the test substance. The identification code is constructed in the following manner:

- The code consists of three parts separated by dashes with an AAAA-XX-YYYY format.
- AAAA is the code for the source of the sample.
- XX is the code for the year of generation or procurement.
- YYYY is either the sequential or randomly-selected unique number for the sample.

After assigning an identification code, give a short description of the sample. On the "Results Form," have the laboratory technician enter information that uniquely identifies a test: a four-letter code for the research laboratory performing the test, the sample identification code, the test date, the bacterial strain used, an optional strain batch identifier, and an optional identifier for the batch of exogenous activation enzyme mixture when used. Next the technician should enter quality control and quality assurance information such as results of a check for phenocopy colonies, the sterility for the activation mixture and sample, the amount of activation mixture used, the time and temperature of any pre-incubation conditions, and the name of the technician performing the test. If the technician determines that additional comments are needed, he/she should place a 1 in box 64 and record his/her comments at the bottom of the results form. Following that, the technician records the counts of revertant colonies per plate noted for varying doses of test agent, as well as for the positive control and negative control. Within one test, the technician may enter a maximum of 24 doses (by using the "Results Continuation Form") with up to 5 plates at each dose level. (The design of these forms was based on those in use at the National Cancer Institute [NCI].)

To provide good quality assurance data on the activation mixtures used, use the two forms provided to record essential and useful information. The first form records information concerning the preparation of the activation system. Included is information concerning: (1) the supplier, if other than the research laboratory, (2) the animal(s) used, (3) the type of induction, if any, used, and (4) the details of preparation. The second form records any quality control information gathered. These forms are based on former work completed at the National Institute for Environmental Health Sciences (NIEHS) and previous efforts by NCI and the National Center for Toxicology Research (NCTR).

SECTION 10

REPORTING AND ANALYSIS OF TEST RESULTS

Three report formats are currently available that list the entire data base or selected subsets. The "Plate Count Report" lists individual plate counts, mean, and standard deviation at each dose level on a test-by-test basis. Tests are grouped in order by test agent, sample code, and research laboratory performing the test. The user may select the limits of this report by specifying a research laboratory and/or ranges of sample identification codes. The "Plate Count Summary Report" groups test results for reporting on an experiment-by-experiment basis. An experiment is defined as all of the tests performed on a single day in a particular research laboratory. The user has the option of limiting the scope of this report according to research laboratory and/or experiment date. For example, he/she can easily request that only experiments performed between January 1, 1979 and January 10, 1979 by laboratory XXXX be included in the report. The "Statistical Summary" page displays the raw data for a test and various statistics pertaining to the test results. Next to this information appears a graph of plate counts versus test agent dose level. The curved line is generated by fitting a linear model to the observed plate counts.

The "Plate Count Report" and the "Plate Count Summary Report" are designed for output to a CRT screen and an attached hard-copy unit. Data from the "Statistical Summary" also can be output via a standard line printer. Requests for reports and specifications of data selection criteria can be done from an inactive terminal if desired.

Experimental data selected by this program can also be input to another program, GRAPH60, which generates CRT graphs that display dose versus mean plate count for multiple microorganism strains or test samples within an experiment.

PART III

EXPERIMENT PLANNING, SAMPLE INFORMATION FORMS, AND COMPUTERIZATION PROCEDURES

SECTION 1

REPORTING OF TESTING INFORMATION

To expedite experiment planning, information handling, and data analysis, a series of forms has been developed to help guide the investigator in providing permanent records and creating a permanent data storage and analysis system. Some of these forms allow for the computerization of information.

TERMINOLOGY

To prevent confusion concerning the procedures and levels of effort necessary for various tasks, three words have been assigned special meanings. An explanation of these three words -- "test," "experiment," and "study" -- will allow easier dialogue among investigators, managers, and those responsible for the computerization of information.

For efforts within the HERL Genetic Bioassay Branch (GBB), a "test" refers to the response of one sample (1) tested with one strain of an indicator organism, (2) using either exogenous or endogenous activation, (3) performed in one laboratory, (4) performed upon a single date, and (5) providing only one dose-response curve.

An "experiment" refers to all tests performed in one laboratory in a single day. Likewise, a "study" is a series of experiments designed to answer a single research question.

STUDY PLANNING AND ORDER OF CUSTODY INFORMATION

Items contained on the "Study Planning and Order of Custody Information Form" used for GBB information are to be completed in the following manner:

Page 1

- Date: Give the date on which the planned study was finalized and the form completed.
- Lead Laboratory: State the laboratory that assumes the primary responsibility for completion and publication of this study. This may or may not be an EPA laboratory. Use an appropriate four-letter abbreviation when possible (Table A-2).
- Lead Planner: Give the initials and last name of responsible person from lead laboratory.
- Other Planners: List up to four other planners involved in the study plan (initials and last name of each).
- Title: Provide brief description of the study (up to 140 characters).
- Purpose (Hypothesis): State a null hypothesis. For example, the null hypothesis may be: "There is no difference in the mutagenicity of the five combustion source organics sampled.". (Up to 210 characters.)
- How do the samples differ from one another?: Provide a general viewpoint answer to this question (e.g., "Samples differ according to level of NO_x in dilution air."). (Up to 210 characters.)
- Control Samples Needed: List control samples needed in each part of the study for a complete study (up to 210 characters).

- How are the data to be used?: Briefly answer to whom and for whom the results will be supplied (up to 210 characters).
- Special Concerns/Comments: Provide any additional description and comments (up to five 70-character lines).

Page 2

- Persons Responsible for Acquiring Samples: Enter initials and last names of up to three persons responsible for acquiring the samples for this study. Note their organization and phone number. (Use FTS numbers for government employees.)
- Samples to be Tested: For each sample enter:

  (1) Description (up to 50 characters)

  (2) Initials of person responsible (3 characters)

  (3) Sample number (10 characters, XXXX-YY-NNNN, where XXXX is laboratory, YY is year, and NNNN is sequential number).

This form should be submitted for entry into a computerized file. A copy of the form and any attached notes and information should be placed into a notebook labeled with the study number and title.

STUDY PLANNING AND ORDER OF CUSTODY INFORMATION FORM (page 1)

Date: ______________________________

Lead Laboratory: ______________________________

Lead Planner: ____________________

Other Planners: ____________________

____

____

____

STUDY NUMBER:

____

Title (Brief but Descriptive): ______________________________

____

____

Purpose (Hypothesis): ______________________________

____

____

____

How do samples differ from one another?: ______________________________

____

____

Control Samples Needed: ______________________________

____

Date of Previous Associated Experiment: ______________________________

How are the data to be used?: ______________________________

____

____

____

____

____

Special Concerns in Design of Experiment/Comments: ____________________

____

____

____

** ATTACH ANY APPROPRIATE WRITE-UPS AND NOTES **

STUDY PLANNING AND ORDER OF CUSTODY INFORMATION FORM (page 2)

A. Persons Responsible for Acquiring Samples:

PERSON	ORGANIZATION	PHONE

B. Samples to be Tested (Including Controls, Blanks, etc.):

	SAMPLE	PERSON	SAMPLE NUMBER
1.			
2.			
3.			
4.			
5.			
6.			
7.			
8.			
9.			
10.			
11.			
12.			
13.			
14.			
15.			
16.			
17.			
18.			
19.			
20.			

** ATTACH SAMPLE INFORMATION SHEETS **

SAMPLE INFORMATION FOR CHEMICALS

This form was designed for recording information on individual chemicals, not complex mixtures. Items should be completed in the following manner for each chemical:

- Name of Substance: Give the name used by the laboratory investigator (up to 50 characters).
- Chemical Abstract Service (CAS) Registry Number: Enter the CAS registry number, which can be obtained from the Environmental Mutagen Information Center (EMIC) or the Chemical Information Service (CIS).
- Molecular Formula: Enter the molecular formula as a continuum, e.g., C20H1002N (up to 30 characters). The formula may be obtained from the CIS or the Merck Index.
- Warnings/Precautions Needed: Enter any warning pertinent to safety, e.g., explosive at room temperature, refrigerate at 4°C (up to 3 lines of 70 characters each).
- Antidote & Emergency Treatment: Enter information needed if a spill should occur or source of information if available immediately within the laboratory (up to 10 lines, 70 characters/line).
- Source/Supplier: Enter name and address of the chemical's supplier (up to 6 lines, 70 characters/line).
- Form on Receipt: Check appropriate box and enter concentration if appropriate.
- Storage on Receipt: Enter the place where and temperature under which the chemical was stored (50 characters).
- Form for Testing: Check appropriate box and enter concentration if appropriate.
- Suggested Method of Storage: Enter how the sample as a test substance should be stored (50 characters).
- Amount Sent for Testing: Enter total amount of chemical in units.
- Investigator's Sample Number: May enter any sample number other than computerized number.

- Computer Number: Enter the GBB-assigned number.
- Date: Enter date of receipt.

After completing the form, submit it for entry into the computer system. Enter a copy of the form and attached notes and information into an appropriate notebook.

Date: ______________________

SAMPLE INFORMATION FOR CHEMICALS FORM

Name of Substance: ______________________

CAS Registry Number: ______________________

Molecular Formula: ____________ Molecular Weight: ____________

WARNINGS/PRECAUTIONS NEEDED: ______________________

ANTIDOTE & EMERGENCY TREATMENT: ______________________

SOURCE/SUPPLIER: ______________________

Form on Receipt: ☐ GAS ☐ LIQUID ☐ SOLID ☐ SOLUTION (CONCENTRATION: ________)

Storage on Receipt: ______________________

Form Sent for Testing: ☐ GAS ☐ LIQUID ☐ SOLID ☐ SOLUTION (CONCENTRATION: ________)

Suggested Method of Storage: ______________________

Amount Sent for Testing: ______________________

Investigator's Sample Number: ______________________

** ATTACH ANY ADDITIONAL INFORMATION **

Investigator: ______________ Signature: ______________

Assigned Computer Number: ______________________

SAMPLE INFORMATION FOR COMPLEX MIXTURES

"Sample Information Sheet for Complex Mixtures" form should be completed at the time of the collection process for each sample to be submitted.

- Assigned Computer Number: Enter the GBB 10-digit number.
- Investigator ID Number(s): Enter the identifying number(s) used for the compound (other than the Assigned Computer Number).
- Type of Number: Enter, for example, the dynamometer run number.
- SIC/Census Number: Enter the descriptive number from SIC and/or Census Bureau manuals.
- Expansion Code: See Appendix C, THE COMPLEX SAMPLE EXPANSION CODE.
- Collection Site: Give the address and description of the collection site (4 lines, 70 characters each).
- Collection Date: Enter the date(s) on which the sample(s) was (were) collected.
- Type of Collection Device: Give the number of collection devices and the type of collection media used. Answer inquiries that describe the collection device (50 characters each).
- Number of Media Units Used: Enter the number of units used.
- Source(s) Sampled: Describe the type of source(s) being sampled (4 lines, 70 characters each).
- Operating Modes, etc.: Describe the source at the time of collection (7 lines, 70 characters each).
- Sample Preparation for Bioassay to be Performed by: Give the name and address of institution performing bioassays (4 lines, 70 characters each).
- Chemical Analysis to be Performed by: Give the name and address of the institution performing chemical analyses (4 lines, 70 characters each).

- Special Handling Instructions: Enter useful information on performance of bioassay or chemical analysis (4 lines, 70 characters each).

Submit completed form for computer entry. Enter copy of form and attached notes and information into a proper notebook.

Date: ____________________

SAMPLE INFORMATION FOR COMPLEX MIXTURES FORM

Collection Information

Assigned Computer Number: ____________________
Investigator ID Number(s): ____________________
Type of Number (Filter No., Run No., etc.): ____________________
(See back if listing appropriate)

SIC/Census Number: ____________________

Expansion Code for SIC/Census Bureau Number: ____________________

Collection Site: ____________________
____________________(Attach map if necessary)

Collection Date(s): ____________________

Type of Collection Device: ____________________
Number of Collection Devices Used: ____________________
Type of Collection Media (Glassfiber, Tenax, etc.): ____________________
____

Number of Media Units Used: ____________________
____
____

Source(s) Sampled: ____________________
____
____

Operating Modes, Fuels, Test Cycles, Test Conditions of Source: ____________________
____
____
____

Sample Preparation for Bioassay to be Performed by: ____________________
____________________(Attach instructions)

Chemical Analysis to be Performed by: ____________________
____________________(Attach instructions)

Special Handling Instructions: ____________________
____
____

** ATTACH ANY ADDITIONAL INFORMATION SHEETS **

Investigator: ____________________ Signature: ____________________

SAMPLE PREPARATION FOR BIOASSAY

Enter the following information as described:

- Sample Number: Enter the GBB computer number.
- Date Sample Received: Enter the date received by chemistry laboratory for processing.
- Date Instructions Received: Enter the date on which instructions are received by the chemistry laboratory.
- Method of Storage Before Preparation: (a) Form: Form of sample (e.g., on filter, as a particulate, as sludge, etc.). Check or supply other information as needed (4 lines, 70 characters each).
- Date of Preparation: Provide date on which preparation of the sample was completed (20 characters).
- Extraction/Elution Method: Give a brief description of the extraction/elution method used (2 lines, 70 characters each).
- Solvents Used: List the common names of the solvents used.
- Concentration Method: Give a brief description of the concentration method used (3 lines, 70 characters each).
- Total Amount of Sample Recovered: Enter needed amounts and units of sample recovered.
- Material Sent As: Check and supply information as needed.
- Date Sent to Bioassay Laboratory: Enter date sample sent from chemistry laboratory.
- Chemical Analysis Information: Check as appropriate.
- Notes: Enter any additional information (3 lines, 70 characters each).
- Investigator: Enter the name of person doing tne preparation.
- Assigned Computer Number: Enter number assigned to the sample.

Sign the form after its completion and submit it for computerization.

Place a copy of the form and any additional notes in an appropriate notebook.

Date:

SAMPLE PREPARATION FOR BIOASSAY FORM

Sample Number:

Date Sample Received:
Date Instructions Received (attach):
Method of Storage Before Preparation:

a. Form:
b. Temperature: Room Refrigerator (__°C) Freezer (__°C)
c. Other:
d. Other Information: Yes (Attach) No

Date of Preparation:

Extraction/Elution Method:

Solvents Used:

Concentration Method (Solvent Removal/Exchange Method):

Total Amount of Sample Recovered:
Percent Extractable: %

Material Sent As:

GAS, Cylinder size, etc.:
LIQUID, Amount:
SOLID/TAR, Weight:
SOLUTION, Concentration: Amount:
Solvent:

Date Sent to Bioassay Laboratory:

Chemical Analysis Information: Attached/On back Forthcoming
Not Done Unavailable/Lost

Notes:

Investigator: Signature:

Assigned Computer Number:

** ATTACH ANY ADDITIONAL INFORMATION SHEETS **

BIOASSAY SAMPLE STORAGE INFORMATION

Supply the requested information as directed below on the "Bioassay Sample Storage Information Form."

- Computer Number: Enter the GBB-assigned number.
- Lot Number: Enter a non-GBB number.
- Known to be previously tested?: Refers to similar or same sample under another GBB number.
- Source: Enter the supplier from whom the sample was received.
- Received by: Enter the initials or name of person personally receiving the sample. Also enter the date sample was received.
- Description of Material on Receipt: Check or supply appropriate information.
- Source of Other Information:
- Sample Storage on Receipt of Information:
- Usage: Tracking system for sample usage (for Technician's notebook).
- Notes: Enter additional information.

Date: ______________________

BIOASSAY SAMPLE STORAGE INFORMATION FORM

Computer Number: ______________________

Lot Number (Run No., etc., if any): ______________________

Known to be tested previously? ☐ Yes ☐ No

If yes, last previous test date: ______________________

Last previous test laboratory: ______________________

Source (person, laboratory, supply house, etc.):

____

____

____

____

____

Received by: ______________________ Date: ______________________

Description of Material on Receipt:

a. Name Description: ______________________

____

b. (1) Solid: ☐ crystals ☐ powder ☐ tar-like

(2) Solid in Solution: ☐ fluid ☐ viscous ☐ suspension
☐ other (______________________)

(3) Liquid: (a) ☐ fluid ☐ viscous
(b) ☐ clear ☐ translucent ☐ opaque

(4) Gas ☐ : Method of Containerization: ______________________

____

c. Color: ______________________

d. Noticeable Odor: ☐ Yes ☐ No

If yes, please describe: ______________________

____

e. Any Further Description: ______________________

____

____

** ATTACH ANY ADDITIONAL INFORMATION SHEETS **

BIOASSAY INSTRUCTIONS

Supply the information indicated below on the "Bioassay Instructions Form."

- Sample Numbers: Indicate the range of sample numbers involved.
- Experimental Design by: Enter the investigator's name and phone number.
- Test Laboratory: Enter the laboratory performing the bioassay.
- Responsible Investigator: Enter the name of the investigator performing the bioassay.
- Solvent for Testing -- Notes: Check or supply needed information.

Send the original of this form to the testing laboratory and send one copy for entry into the computer. Also place one copy in the proper notebook.

Date: ____________________

BIOASSAY INSTRUCTIONS FORM

Sample Number(s): ____________________
Experimental Design by: ____________________
Phone: ____________________
Test Laboratory: ____________________
Responsible Investigator: ____________________
Solvent for Testing: ____________________
Approximate Amount Available
for Testing: ____________________

Test(s) to be Performed:

☐ Plate Incorporation
☐ Pre-incubation
☐ Fluctuation
☐ Host-mediated
☐ Other: ____________________

☐ Spot
☐ Suspension
☐ Co-cultivation
☐ Well

Conditions for Test:

a. Needed Comparisons: ____________________
____
____

b. Strains: ______ > ______ > ______ > ______ > ______
______ > ______ > ______ > ______ > ______

c. Doses: 1. ______ 2. ______ 3. ______ 4. ______ 5. ______
6. ______ 7. ______ 8. ______ 9. ______ 10. ______

d. Replicates Per Dose: ____________________

e. Initial Concentration: ____________________

f. Activation: ☐ +/- S-9 ☐ +S-9 ☐ -S-9 ☐ Other*
(Attach to back) ☐ Aroclor 1254-Induced ☐ Uninduced ☐ Other
☐ Ames ☐ In-house ☐ Other*

g. Additional Information (e.g., add β-glucuronidase): ____________________
____

Notes: ____________________
____
____

Investigator: ____________________ Signature: ____________________

SECTION 2

HERL INTERIM *IN VITRO* SYSTEM USER FORMS COMPLETION INSTRUCTIONS

INTRODUCTION

Design of the "Results Entry Form," the various code tables, and the coding conventions presented here was directly patterned on forms and form completion instructions developed by Ms. Mary Linhart of the National Cancer Institute for NCI's *in vitro* system.

The Health Effects Research Laboratory in Research Triangle Park, North Carolina, developed the MUTAG computer system to systematize results obtained from *in vitro* tests conducted by the Genetic Toxicology Division and its contractors. The laboratory has written a series of computer programs to allow verification of experimental results, statistical analysis of data, and collection of reports of raw data.

Experimental results are collected on forms, keypunched, and input into the MUTAG network of computer programs. Currently, the system is designed to handle only results from the bacterial plate incorporation tests. It will be modified in the future to handle results from a variety of tests.

All data should be entered on forms by the technicians conducting the experiments. Blank forms can be obtained by photocopying old forms, or by contacting Ms. Dorothy Denning or Ms. Bonita Hodges, Data Preparation and Control Section, MD-55, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711. When using the three-part, self-carbon forms, submit the top white sheet to Ms. Denning. Retain the other two parts for laboratory records. When a contractor company submits keypunched data (cards or tape), it should submit one copy (any color) of the forms also. Any questions concerning how to complete a form or submit data may be addressed to Ms. Carol Evans (919-541-2567 or FTS 629-2567).

GENERAL INSTRUCTIONS

At the present time, five user forms are required: the results form, the results form's continuation sheet, the sample ID form, and two activation batch preparation forms.

All written entries should be clear and dark.

To assure the collection of uniform data, code number entries are required for many items. For user convenience, the codes have been printed on the forms when possible. Some items require reference to the Code Tables in this manual for completion (Appendix A). If the Code Tables do not contain an appropriate code for the response, use the "OTHER" code and write the response in the REMARKS section of the form. Carefully identify the item to which the remarks pertain.

On every form, the individual completing the form should note his/her initials in the blocks in the "Forms Completion" area at the bottom right of the sheet, and should make note of the form's completion date as well.

HERL *IN VITRO* RESULTS FORM

Key Items

The items on the top line of the form (Research Lab ID through Microorganism Strain) uniquely identify a test and must be completed. (Please note the exception for Microorganism Batch Number.)

Top Line

- Update Code: Do not complete. This item is for use by HERL data processing personnel only.
- Research Lab ID: Enter the complete four-letter code from the table of valid research laboratory IDs which identifies you organization (Table A-2). This code identifies the group that is conducting the experiment.
- Experiment Date: Enter the date the experiment was initiated. Use the standard MONTH/DAY/YEAR notation.
- Test Sample Identification: Enter the full 10-character sample identification number assigned by EPA which identifies the agent being tested. This number consists of a four-letter sample preparation laboratory code, followed by a two-digit number for year of preparation, followed by a four-digit number.
- Activation Batch: If the results reported on this form refer to a test done without activation, enter a "-" left-justified (Column 25). If the results refer to a test done with activation, enter an activation preparation batch identifier in positions 25 through 30. It is recommended that the first three characters be used as follows:

1st character R = rat
M = mouse
H = hamster
B = bovine
G = guinea pig
D = dog
A = rabbit

2nd character L = liver
U = lung
K = kidney
T = testes
B = bladder
S = skin
E = serum/blood

3rd character I = Induced, compound not specified
N - Non-induced
A = Aroclor-induced
F = Naphthoflavone
P = Phenobarbitol
Q = Naphthoflavone plus phenobarbitol
M = 3-Methylcholanthrene
Z = Aroclor plus phenobarbitol

The remaining three characters may contain a unique numeric identifier. Thus, RLI001 is the first rat liver-induced activation preparation for a testing laboratory (research laboratory ID). RLI001 is the second rat liver-induced activation preparation, etc. If this information is not known, enter a "+" in Column 25 and leave the other columns (26-30) blank. If a test is done both with and without activation, two forms must be completed.

- Test Type: Enter the appropriate test type code from Table A-14.

- Microorganism Strain: Enter the appropriate strain abbreviation from Table A-13. If the strain abbreviation is less than six characters, enter it left-justified starting in Column 33.

- Microorganism Strain Batch: Enter a four-character microorganism strain batch identifier or leave blank if the batch is not known.

Second Line

- Remarks Made: If any remarks concerning the circumstances of this test were made in the REMARKS area at the bottom of the form, enter a code of "1" for "yes." If no remarks were made, enter a "2" or leave blank for "no."

- Phenocopy Check Conclusion: Enter the appropriate code from Table A-17 to identify the conclusion concerning the results of restreaking (or replicate plating) when colonies were restreaked to check for phenotypic reversion.

- Sterility S-9 Mix: Using the codes that appear on the form, enter the results of checking the sterility of the S-9 mixture. "CONTAM" stands for "CONTAMINATED."
- Activation Mixture Per Plate: Enter (right-justified) the amount of activation mixture placed on each plate. The amount must be reported in microliters. If activation mixture was not used, leave item blank.
- Pre-incubation Time: If the test type indicated pre-incubation, enter the duration of incubation in minutes (right-justified). Otherwise, leave this item blank.
- Pre-incubation Temperature: If the test type indicated pre-incubation, enter the temperature of incubation in degrees centigrade. Otherwise, leave this item blank.
- Technician: Enter the initials of the technician conducting the test. Always supply the same three initials for a technician. If more than one technician has the same initials, assign a unique set of initials to each one (e.g., vary the middle initial to differentiate). If a technician has no middle initial, choose one.

On some of the forms, space has been left for the user to write in information about elements of the experiment (e.g., animal, organ, inducer, microorganism). This space is provided for user convenience; the data will not be entered into the computer master files.

Subsequent Lines (Card Codes B through Y)

Subsequent lines on the form should be used for reporting the plate counts for the positive and negative control(s) and the various dose levels of the test agent. A maximum of 24 doses can be reported by using the continuation form. This form allows for a maximum of five replicate plates at each dose. IMPORTANT: Enter information in the order described below and do not skip lines.

- Solvent/Positive: When reporting solvent (negative control) or positive control plate counts, enter the appropriate code from Table A-15 denoting the substance used as the control agent. For a negative control entry, if no solvent was used, enter a code of "50." If more than one positive or negative control substance was used, enter the appropriate code and data on subsequent lines. Enter all negatives on consecutive lines first, then all positives. Leave this area blank when reporting results for the test agent (sample ID doses).

  Even if the same positive and negative controls were used for a number of tests, the control plate counts must be repeated on each form.

- Units of Concentration: Enter the code as shown on the form to identify the units in which the stock concentration is reported (blank, mg/mL; 2, μg/mL; 3, μL/mL). This item may also be used to identify the units of a dose pre-computed by the technician and entered solely in either the stock concentration or the amount per plate area.

- Stock Concentration: Enter the stock concentration right-justified. Leading zeros are not required. If the dose is in μL or nL (as with a liquid sample), this item may be left blank. When reporting the plate counts for a negative control denoted as "50," this item may be left blank also.

- Amount Per Plate: Enter in μL, right-justified, the amount of liquid plated on an individual plate. This field may be left blank when reporting plate counts for a general negative control (positive/solvent = "50"). If the actual dose level is in μL, enter it here, leaving blank the units of concentration and stock concentrations. If the pre-computed actual dose is in nL, enter it here; enter "3" in units of concentration.

  NOTE: It is desirable to report both stock concentration and amount per plate whenever possible. The computer programs will then calculate the actual dose per plate in the correct units.

- Plate Counts A through E: Enter the number of colonies actually counted on each plate. Enter the number right-justified; leading zeros are not required. If a plate was counted at zero, enter a "0." Do not leave it blank. Other legitimate entries are:

  TNTC - too numerous to count
  NATC - not able to count
  T* - toxic

  If less than five plates were counted, leave non-used plate counts blank. Enter plate counts starting at A, then proceed to B, etc.

- BG (Background Code): Enter the numeric code from Table A-16 which designates the appearance of the background lawn of cells for the corresponding plate. If this item is left blank, the computer operator assumes that the background lawn was observed to be normal. The experimenter may instead use this item to refer to a comment about the plate in the REMARKS area at the base of the form by entering any single letter (starting with "A"). In the REMARKS area, enter the letter(s) chosen and the associated comment. (Enter the letter next to each plate count to which the comment applies.) For example: (A) -Plate count is inaccurate due to . . . .

Remarks

Enter here any remarks concerning items on the form. When the remark refers to a plate count(s) and Table A-16 does not contain an appropriate description, use a letter in the background code box, as described above. Do not refer to an item code or card code in specifying your comment since these items are not computerized. Remarks pertaining to information reported on the continuation form should be reported here also. If space is too limited, attach a blank sheet of paper and continue. Keep remarks clear and concise when possible, since they will also be keypunched.

Continuation Form

The "Results Continuation Form" should be used when the nine dose lines on the original form will not accommodate all the controls and doses for a particular test. The top line of the continuation sheet should be completed to match the top line of the first results form. The two sheets should then be stapled together and submitted as a unit.

HERL IN VITRO RESULTS FORM

1 ☐ UPDATE CODE	2-4 I V R SYSTEM ID	5-8 ☐☐☐☐ RESEARCH LAB ID	9-14 ☐☐ ☐☐ ☐☐ MO DA YR EXPERIMENT DATE	15-18 ☐☐☐☐ LAB 19-20 ☐☐ YR 21-24 ☐☐☐☐ NUMBER	25-30 ☐☐☐☐☐☐ ACTIVATION BATCH	31-32 ☐☐ TEST TYPE (Table A-14)	33-38 ☐☐☐☐☐☐ STRAIN 39-42 ☐☐☐☐ BATCH NO MICROORGANISM

ANIMAL ORGAN INDUCER MICROORGANISM	Ⓐ 64 ☐ REMARKS MADE? YES = 1	Ⓑ 65 ☐ PHENOCOPY CHECK CONCLUSION (Table A-17)	Ⓒ 66 ☐ STERILITY S9 MIX 1 NOT CONTAM 2 CONTAM 3 NOT CHECKED	Ⓓ 67-70 ☐☐☐☐ ACTIVATION MIXTURE PER PLATE (µL)	Ⓔ 71 ☐ SAMPLE STERILITY CHECK 1 NOT CONTAM 2 CONTAM 3 NOT CHECKED	Ⓕ 72-73 ☐☐ TIME (min) 74-75 ☐☐ TEMP (cent) PRE-INCUBATION	Ⓖ 76-78 ☐☐☐ TECHNICIAN	80 A CARD CODE

	DOSE LEVEL			PLATE A		PLATE B		PLATE C		PLATE D		PLATE E		
Ⓗ SOLVENT POSITIVE (Table A-15)	Ⓘ UNITS OF CONCENTRATION Blank mg/ml 2 µg/ml	Ⓙ STOCK CONCENTRATION	Ⓚ AMT. PER PLATE µL)	Ⓛ COUNT	Ⓜ BG	Ⓝ COUNT	Ⓞ BG	Ⓟ COUNT	Ⓠ BG	Ⓡ COUNT	Ⓢ BG	Ⓣ COUNT	Ⓤ BG	CARD CODE
43-44	45	46-50	51-54	55-58	59	60-63	64	65-68	69	70-73	74	75-78	79	80
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REMARKS: INDICATE ITEM CODE AND CARD CODE

FORMS COMPLETION

INITIALS ☐☐☐

HERL IN VITRO RESULTS FORM

1 UPDATE CODE	2-4 SYSTEM ID	5-8 RESEARCH LAB ID	9-14 EXPERIMENT DATE (MO DA YR)	15-18 LAB	19-20 YR	21-24 NUMBER	25-30 ACTIVATION BATCH	31-32 TEST TYPE (Table A-14)	33-38 STRAIN (MICROORGANISM)	39-42 BATCH NO (MICROORGANISM)
	IVR	NSNC	01 12 81	ARBX	81	0001	RL1006	01	TA1538	

ANIMAL

ORGAN

INDUCER

MICROORGANISM

Ⓐ 64 REMARKS MADE? YES = 1	Ⓑ 65 PHENOCOPY CHECK CONCLUSION (Table A-17)	Ⓒ 66 STERILITY S-9 MIX 1 - NOT CONTAM 2 - CONTAM 3 - NOT CHECKED	Ⓓ 67-70 ACTIVATION MIXTURE PER PLATE (μL)	Ⓔ 71 SAMPLE STERILITY CHECK 1 - NOT CONTAM 2 - CONTAM 3 - NOT CHECKED	Ⓕ 72-73 TIME (min) PRE-INCUBATION	74-75 TEMP (cent) PRE-INCUBATION	Ⓖ 76-78 TECHNICIAN	80 CARD CODE
1	1	1	500	3			XAB	A

	DOSE LEVEL			PLATE A		PLATE B		PLATE C		PLATE D		PLATE E		CARD CODE
Ⓗ SOLVENT POSITIVE (Table A-15) 43-44	Ⓘ UNITS OF CONCENTRATION Blank mg/ml 2 μg/ml 45	Ⓙ STOCK CONCENTRATION 46-50	Ⓚ AMT. PER PLATE μL 51-54	Ⓛ COUNT 55-58	Ⓜ BG 59	Ⓝ COUNT 60-63	Ⓞ BG 64	Ⓟ COUNT 65-68	Ⓠ BG 69	Ⓡ COUNT 70-73	Ⓢ BG 74	Ⓣ COUNT 75-78	Ⓤ BG 79	80
SOL 51		1.00	500	25		10		15						B
POS 02	2	5.00	100	850		750		700						C
04	2	5.00	100	620		640		609						D
		10.00	10	510	C	300	C	222	C					E
		10.00	40	706		712		TNTC	6					F
		10.00	90	900		NATC	A	907						G
		10.00	200	NATC	A	1000	B	1025						H
														I
														J

REMARKS: INDICATE ITEM CODE AND CARD CODE

Forgot to check sample sterility
(A) Background too cloudy to count
(B) Background was very cloudy - count may be inaccurate
(C) Wide count variation not explainable

2/20/81

FORMS COMPLETION
INITIALS XAB

HERL IN VITRO RESULTS CONTINUATION FORM

1 ☐ UPDATE CODE	2-4 I V R SYSTEM ID	5-8 ☐☐☐☐ RESEARCH LAB ID	9-14 ☐☐ ☐☐ ☐☐ MO DA YR EXPERIMENT DATE	15-18 ☐☐☐☐ LAB	19-20 ☐☐ YR	21-24 ☐☐☐☐ NUMBER	25-30 ☐☐☐☐☐☐ ACTIVATION BATCH	31-32 ☐☐ TEST TYPE (Table A-14)	33-38 ☐☐☐☐☐☐ STRAIN MICROORGANISM	39-42 ☐☐☐☐ BATCH NO

	DOSE LEVEL			PLATE A		PLATE B		PLATE C		PLATE D		PLATE E		
Ⓗ SOLVENT POSITIVE (Table A-15)	Ⓘ UNITS OF CONCENTRATION Blank mg/mL 2 µg/mL	Ⓙ STOCK CONCENTRATION	Ⓚ AMT. PER PLATE (µL)	Ⓛ COUNT	Ⓜ BG	Ⓝ COUNT	Ⓞ BG	Ⓟ COUNT	Ⓠ BG	Ⓡ COUNT	Ⓢ BG	Ⓣ COUNT	Ⓤ BG	CARD CODE
44-45	43	46-50	51-54	55-58	59	60-63	69			70-73	74	75-78	79	80
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Error Corrections

Occasionally, changes will be required on forms already submitted; for example, correction of a transcription mistake, adding a dose line for a test which was accidentally omitted, or removing blocks of data that are invalidated for some reason.

Changes can be made in the following manner:

- Make a photocopy of the form containing the erroneous data.
- Using a distinct color of ink (e.g., red), mark out the erroneous data. Then place the correct values just above the old data.
- To clarify or further describe the correction or addition being made, place a note in the REMARKS area.
- Clearly write the word "UPDATE" at the top of the form.
- If the form on which the incorrect data were previously submitted cannot be located, fill out a new form. Clearly write the word "UPDATE" at the top.
- Always complete the top two lines to identify the test. If the change or addition involves items in the top line, the first method of correction is preferable. If not possible, note the original contents of the top line in the REMARKS area.
- If the change involves one (or more) of the dose lines, fill out completely all items starting with the card code "B:" line. Then, in the REMARKS area, note whether or not the dose line entered is to be "DELETED," "ADDED," or be a "REPLACEMENT" for a previously reported dose line. If the line entered is a replacement for a previously reported dose line, identify the line desired replaced in the REMARKS area.

An alternate method may also be used. If a report of test results that have been entered into the computer files is received, corrections may be noted in a clearly visible color of ink. Mail a copy of the corrected report sheets to Ms. Denning. Currently, two report formats exist. The plate count report lists results on a test-by-test basis, but does not include all items that were entered on the results form. The quality assurance listing dumps test results from the master computer file in a format similar to the original results form.

SAMPLE IDENTIFICATION FORM

The "Sample Identification Form" provides for registry of a chemical test sample code as a valid test agent. It also allows the user to specify a chemical name or other descriptive title (up to 41 characters) to be used in report headings. Use of a sample ID Code on a results form which has not been registered via this form will delay the entry of the test results into the computer files.

The sample identifier is assigned by the EPA laboratory or analysis group that furnishes a test agent sample to the testing laboratory. It is formed as follows:

Pos. 1 - 4	Valid sample preparation lab code from tables
5 - 6	Preparation year
7 - 10	Unique number

HERL IN VITRO SYSTEM
SAMPLE IDENTIFICATION FORM (INTERIM)

SYSTEM ID
I V S
2 - 4

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

LAB 5-8 – SAMPLE ID 9-10 – NO 11-14 SAMPLE DESCRIPTION 15-56

FORMS COMPLETION
INITIALS

HERL IN VITRO SYSTEM
SAMPLE IDENTIFICATION FORM (INTERIM)

SYSTEM ID: IVS (2-4)

LAB (5-8)	SAMPLE ID (9-10)	NO (11-14)	SAMPLE DESCRIPTION (15-56)
ARBX	81	0001	RAINWATER - HILLSBOROUGH ST
ARBX	81	0026	DIETHYLSTILBESTROL

FORMS COMPLETION

INITIALS ☐☐☐

HERL INTERIM *IN VITRO* SYSTEM ACTIVATION BATCH FORMS

Activation Batch Preparation Form

The purpose of the "Activation Batch Preparation Form" is to provide an accurate record of the preparation of exogenous activation batches to be used in *in vitro* mutagenesis testing.

The following instructions should be carefully followed. Items marked with an asterisk (*) are required.

- UPDATE CODE: Leave blank.
- RESEARCH LAB*: Enter the code of the laboratory to receive the activation batch and to use the batch in testing. All four spaces should be filled in with the appropriate code number from the Supplier/Research Laboratory Code Tables (Tables A-1 and A-2).
- ACTIVATION BATCH: Enter the unique six-character ID number assigned independently by each supplier for each batch of activation preparations. The following format is preferred:

  1st character
  R = rat
  M = mouse
  H = hamster
  G = guinea pig
  D = dog
  A = rabbit
  B = bovine

  2nd character
  L = liver
  U = lung
  K = kidney
  T = testes
  B = bladder
  S = skin
  E = serum/blood

  3rd character
  I = Induced, compound not specified
  N = Non-induced
  A = Aroclor-induced
  P = Phenobarbitol-induced
  F = Naphthoflavone

3rd character (continued)

Q = Naphthoflavone plus phenobarbitol
M = 3-Methylcholanthrene
Z = Aroclor plus phenobarbitol

If the researcher desires to add other letters to designate another type of induction, he/she should contact EPA's Health Effects Research Laboratory.

The remaining three characters may contain a unique numeric identifier. Thus, RLI001 is the first rat liver-induced activation preparation for a contract. RLI002 is the second rat liver-induced activation preparation, etc.

The supplier should provide EPA with the coding scheme employed, if the recommended one cannot be used.

• SUPPLIER*:	Enter the name of the laboratory that prepares the activation batch. Use the codes on the Supplier/Research Laboratory Code Tables (Tables A-1 and A-2).
• TECHNICIAN*:	Give the three-letter indicator of the person directly responsible for preparing the activation batch. The supplier should provide EPA with a listing of these individuals and their initials.
• SPECIES/STRAIN*:	Write out the full species/strain name of the animals used and place the appropriate two-digit code for this species in the code box (e.g., Rat/Fischer, 50). See Table A-18 for additional listings.
• ANIMAL SEX*:	Place either an M (male), F (female), or B (both) into the code box.
• AVERAGE ANIMAL WEIGHT*:	Enter right-justified the numerical units in boxes 25-28. Boxes 29-30 are for the units code from Table A-4 (e.g., 0205, GM).
• DATE INDUCED/ NOT INDUCED*:	If animals are induced, enter the date they are induced; otherwise, place an "X" in the box labeled NOT INDUCED.

• INDUCER*:	If animals are induced, write out the name of the inducer and enter the two-digit code for the inducer (e.g., Aroclor, 05). See Table A-7 for other inducers.
• INDUCER SOLVENT:	Write the name of the solvent for the inducer (if any) and enter the code for this solvent from Table A-15 (e.g., Corn oil, 58).
• AMOUNT OF INDUCER PER ANIMAL:	Boxes 42-46 give the numerical record (e.g., 0333.3), and boxes 47-52 give the units (e.g., UG/KG) for the amount of activation compound used per animal. The units should also be written out in order to prevent confusion (see Table A-4).
• ROUTE OF ADMINISTRATION:	Write out the route of administration and enter the code for this method in boxes 52-53. See Table A-19 for the list of methods.
• DATE FOOD REMOVED:	If food is removed prior to sacrifice, enter the date in standard month-day-year fashion (e.g., 071780 for July 17, 1980).
• DATE ANIMALS SACRIFICED:	Enter the date in standard fashion.
• NUMBER OF ANIMALS SACRIFICED:	Enter right-justified the number of animals sacrificed for the preparation of the activation batch.
• ORGAN DESCRIPTION*:	Write out the name of the organ used and enter the code for that organ (Table A-8) (e.g., Liver, 01).
• POOLED ORGAN WEIGHT:	Place the numerical units in boxes 24-28 (e.g., 1211.5), write out the units (e.g., grams), and enter the units code (e.g., GM). See Table A-5 for units.
• PREPARATION OF MIX (METHOD)*:	Enter the code from Table A-10 that best describes the method used.
• PREPARATION SOLUTION:	Enter the correct code from Table A-9.
• METHOD OF HOMOGENIZATION:	Enter the correct code from Table A-11.
• CENTRIFUGE SETTINGS:	Enter the numerical values for temperature and calculated gravitational force (xg, not rpm) (right-justified).

- TOTAL VOLUME OF PREPARATION: Enter the number of mL of activation batch material prepared (right-justified).
- NUMBER OF VIALS OR TUBES PREPARED: Enter the number of aliquots prepared under this activation batch number.
- STORAGE TEMPERATURE*: Enter the numerical value for the temperature at which the activation batch was stored after preparation.
- FORMS COMPLETION*: Enter the initials of the person who completed the form. The supplier should provide EPA with an appropriate list.

NOTE: Any remarks needed should be made at the bottom of the "Activation Batch Quality Control Form."

HERL IN VITRO SYSTEM
ACTIVATION BATCH PREPARATION FORM

UPDATE CODE ☐ (1)
SYSTEM ID I V B (2 - 4)
RESEARCH LAB ☐☐☐☐ (5 - 8)
ACTIVATION BATCH ☐☐☐☐☐☐ (9 - 14)
SUPPLIER ☐☐☐☐ (15 - 18)

TECHNICIAN: ☐☐☐ (19 - 21)

SPECIES/STRAIN ______________________ CODE: ☐☐ (22-23) Rat-Fischer 50 (Table A-18)
Rat-CD 52

ANIMAL SEX (M, MALE; F, FEMALE; B, BOTH): ☐ (24)

AVERAGE ANIMAL WEIGHT:
WEIGHT: ☐☐☐☐ (25-28) UNIT: ☐☐ (29-30) grams – gm (Table A-5)

DATE INDUCED: MO ☐☐ - DA ☐☐ - YR ☐☐ (31-36) NOT INDUCED: ☐ (37) ('X' - Not Induced, Blank - Induced)

INDUCER: _ CODE ☐☐ (38-39) Aroclor 05 (Table A-7)

INDUCER SOLVENT: _ CODE ☐☐ (40-41) Corn Oil 58 (Table A-15)

AMOUNT OF INDUCER PER ANIMAL:
AMOUNT: ☐☐☐☐.☐ (42-46) UNIT: _ _ _ _ _ _ _ _ _ _ _ UNIT CODE ☐☐⧄☐☐ (47-52) UG/ML (Table A-4)
MG/KG

ROUTE OF ADMINISTRATION: _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ CODE ☐☐ (52-53) Subcutaneous 10 (Table A-19)
Intraperitoneal 12

DATE FOOD REMOVED: ☐☐☐☐☐☐ (54-59)

DATE ANIMALS SACRIFICED: ☐☐☐☐☐☐ (60-65)

NUMBER OF ANIMALS SACRIFICED: ☐☐ (66-67)

CARD CODE A (80)

(Duplicate Cols 1-18 For CARD B)

ORGAN DESCRIPTION: _ CODE ☐☐ (22-23) Liver 01 (Table A-8)

POOLED ORGAN WEIGHT:
AMOUNT: ☐☐☐☐.☐ (24-28) UNIT: _ _ _ _ _ _ _ _ _ _ _ _ UNIT: ☐☐ (29-30) grams – gm (Table A-5)

PREPARATION OF S-9 MIX (METHOD): ☐☐ (31-32) 9000 x g supernatant 01 (Table A-10)

PREPARATION SOLUTION: ☐☐ (33-34) 0.15 Potassium Chloride 01 (Table A-9)

METHOD OF HOMOGENIZATION: ☐☐ (35-36) Potter - Elvehjen, motor 02 (Table A-11)

CENTRIFUGE SETTINGS: ☐☐ (37-38) °C ☐☐☐☐☐☐ (39-44) xg

TOTAL VOLUME OF PREPARATION: ☐☐☐☐ (45-48) ML

NUMBER OF VIALS OR TUBES PREPARED: ☐☐☐ (49-51)

STORAGE TEMPERATURE: –☐☐ °C

CARD CODE B (80)

FORMS COMPLETION
INITIALS ☐☐☐

Activation Batch Quality Control Form

The "Activation Batch Quality Control Form" serves to provide an accurate record of standardized information gathered for prepared activation batches. The following instructions should be carefully followed. Items marked with an asterisk (*) are required.

1. IDENTIFICATION OF ACTIVATION BATCH: This information should be filled in by the person preparing the activation batch.

• UPDATE CODE:	Leave blank.
• RESEARCH LAB*:	Enter the name of the laboratory that will use the activation batch in a bioassay. See Supplier/Research Laboratory Tables (Tables A-1 and A-2).
• ACTIVATION BATCH*:	Enter the identifier code assigned by the supplier.
• SUPPLIER*:	Enter the code for the laboratory that prepared the activation batch (Table A-1).

2.-3. STERILITY CONTROL:

2. TECHNICIAN:	Enter the initials of the technician who performed the sterility check.

3. STERILITY:

• MICROLITERS PLATED:	Enter the number of total microliters of activation batch plated on all plates.
• NUMBER COLONIES:	Enter the total number of colonies counted on the sterility plates.

4. LOWERY PROTEIN DETERMINATION:

UG COLUMN:	Enter the number of micrograms of standard protein.
OD COLUMN:	Enter the OD reading for the corresponding UG column.
SAMPLE OD:	Enter the OD form standard amount of sample or a meaned (weighted) OD value for the amount of sample.
SAMPLE MICROLITERS/ML:	Enter the number of microliters of sample to which the sample OD corresponds.
DILUTION:	Enter the dilution factor (e.g., 01 to 0010) used in calculations.

Most of the above items are for the convenience of the investigator and are not required. However, a complete laboratory notebook maintaining all of the required recordings and calculations should be maintained.

For Lowery protein determinations, the following items are required:

MG/ML:	Enter the number of milligrams of protein per milliliter of activation batch used.
TESTED BY:	Enter the initials of the person who performed the Lowery determination.
DATE:	Give the date on which the protein determination was performed.

5.-6. ADDITIONAL BIOCHEMICAL ACTIVITIES THAT CAN BE MEASURED:

ACTIVITY:	Using Table A-12, record the activity measured and the code.
AMOUNT:	Give the numerical recording of activity.

• UNIT:	Give the units for AMOUNT.
• DATE:	Enter the date on which the test was performed.
• TECHNICIAN:	Enter the initials of the technician responsible for test.

7. BIOLOGICAL ACTIVITY: MUTAGENESIS TITRATION CURVE: This may be generated in either the researcher's or supplier's laboratory.

• STRAIN:	Enter the name of the bacterial strain used for the test. See Table A-13. (Justify right).
• AMT S-9: (CARD CODE D)	Give the amount of S-9 (or activation batch) recorded as mg of S-9 protein per plate (generally within a range of 0.1 mg/plate to 5.0 mg/plate.
• MUTAGEN CODE*:	Enter the mutagen code from Table A-15.
• AMOUNT (UGS)*:	Enter the number of micrograms of control mutagen used.
• REVERTANTS PER PLATE:	Give the mean revertant count for each level of S-9 for each mutagen used. Justify the numeric values right. Justify the alphabetic notations left:

T*	toxic
TNTC	too numerous to count
NATC	not able to count

8. REMARKS: Include any clarifying remarks needed for either the "Activation Batch Preparation Form" and/or the "Activation Batch Quality Control Form." Carefully identify the item being discussed. Several examples follow:

• AMOUNT OF INDUCER:	Multiple applications (3) were made. This records the total amount given.
7. BIOLOGICAL ACTIVITY:	Amt S-9: Additional doses of S-9 were also given and are recorded in the laboratory notebook.

5. ACTIVITY: Done according to the protocol of Xavier, *et al.*, Mutation Research 1999.

Use additional sheets if necessary.

9. FORMS COMPLETION: Give the initials of the final person entering data and checking the form before entry.

HERL IN VITRO SYSTEM
ACTIVATION BATCH QUALITY CONTROL FORM

	UPDATE CODE	SYSTEM ID	RESEARCH LAB	ACTIVATION BATCH	SUPPLIER	
1.	☐	I V A	☐☐☐☐	☐☐☐☐☐☐	☐☐☐☐	(DUPLICATE IN ALL CARDS)
	1	2 - 4	5 - 8	9 - 14	15 - 18	

2. TECHNICIAN: ☐☐☐ 19-21   3. STERILITY: MICROLITER PLATED: ☐☐☐☐ 22-25 NUMBER COLONIES: ☐☐☐☐ 26-29

4. LOWERY PROTEIN DETERMINATION:

STANDARD

UG	O.D.
☐☐☐☐ 30-33	☐☐☐☐ 34-37
☐☐☐☐ 38-41	☐☐☐☐ 42-45
☐☐☐☐ 46-49	☐☐☐☐ 50-53
☐☐☐☐ 54-57	☐☐☐☐ 58-61
☐☐☐☐ 62-65	☐☐☐☐ 66-69

CARD CODE A 80

SAMPLE:
O.D. = ☐☐☐☐ 19-22
MICROLITERS/ML: ☐☐☐☐ 23-26
DILUTION: ☐☐ 27-28 TO ☐☐☐☐ 29-32
MG/ML: ☐☐☐☐ . ☐ 33-37
TESTED BY: ☐☐☐ 38-40   DATE: MO ☐☐ - DA ☐☐ - YR ☐☐ 41-46

CARD CODE B 80

5. ACTIVITY: ______________________ CODE ☐☐ 19-20 (TABLE A-12)
AMOUNT: ☐☐☐☐ 21-24   UNIT: _______________
DATE: ☐☐-☐☐-☐☐ 25-30   TECHNICIAN: ☐☐☐ 31-33

6. ACTIVITY: ______________________ CODE ☐☐ 34-53
AMOUNT: ☐☐☐☐ 36-39   UNIT: _______________
DATE: ☐☐-☐☐-☐☐ 40-45   TECHNICIAN: ☐☐☐ 46-48

CARD CODE C 80

7. BIOLOGICAL ACTIVITY: MUTAGENESIS TITRATION CURVE (DO NOT PUNCH IF CARD NOT COMPLETED)

STRAIN(Table A-13): ☐☐☐☐☐☐ 19-24

REVERTANTS PER PLATE

MUTAGEN (Table A-15) CODE:	AMOUNT (UGS)							CARD CODE
	AMT : S-9:	0.0 25-26	☐☐ 27-28	☐☐ 29-30	☐☐ 31-32	☐☐ 33-34	☐☐ 35-36	D
☐☐	☐☐☐.☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	E
☐☐	☐☐☐.☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	F
☐☐	☐☐☐.☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	G
19-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	80

STRAIN: ☐☐☐☐☐☐ 19-24

REVERTANTS PER PLATE

MUTAGEN CODE:	AMOUNT (UGS)							CARD CODE
	AMT: S-9:	0 0 25-26	☐☐ 27-28	☐☐ 29-30	☐☐ 31-32	☐☐ 33-34	☐☐ 35-36	H
☐☐	☐☐☐.☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	I
☐☐	☐☐☐.☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	J
☐☐	☐☐☐.☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	☐☐☐☐	K
19-20	21-24	25-28	29-32	33-36	37-40	41-44	45-48	80

REMARKS:

FORMS COMPLETION
INITIALS ☐☐☐

SECTION 3

OPERATING INSTRUCTIONS FOR THE *IN VITRO* SYSTEM FOR MICROBIAL MUTAGENESIS

INTRODUCTION

This section describes the procedures used to store and retrieve computerized data generated in the *Salmonella typhimurium* plate incorporation assay. If any questions arise concerning the computer system, contact one of the following persons:

Carol Evans
Biometry Division
U.S. Environmental Protection Agency
MD-55
Research Triangle Park, NC 27711
Phone: 919-541-2567

or

Larry Claxton
Genetic Toxicology Division
U.S. Environmental Protection Agency
MD-68
Research Triangle Park, NC 27711
Phone: 919-541-3848

The instructions given in the following program outlines should be followed in the exact order in which they appear.

PROCEDURES COMMON TO ALL RETRIEVALS

1. Power on the TEKTRONIX 4051 (or compatible) terminal and hard copy unit and allow to warm up.
2. Enter: CALL "PRLIST" (to check rate).
3. If rate equals 300 then enter: CALL "RATE",1200,0,2
4. Enter: CALL "TERMIN"
5. Dial 9-549-9192 and once carrier is established, type in UNIVAC (in upper case even parity) and press return.
6. Enter: ID/PASSWORD
7. Enter: @RUN (card information).
8. For the different outputs go to the indicated steps:
    a. Demand mode: Plate count data report, FORTRAN output file or both . . . . B
    b. Demand mode: Plate count summary report and GRAPH60 C
    c. Batch mode: FORTRAN output file . . . . D
    d. Batch mode: Plate count summary report . . . . E
    e. Batch mode: Inventory by sample ID . . . . F

DEMAND MODE: PLATE COUNT, FORTRAN OUTPUT, OR BOTH

1. Enter: @ADD RUNS.ASSAY40
2. Display: ENTER A SINGLE RESEARCH LAB OR ALL

2. (continued)

Response: Choose and enter one of the four-letter codes from the Research Laboratory Code Sheet (Table A-2), e.g., NEIC.

3. Display: `ENTER UP TO 10 SAMPLE IDS OR SAMPLE ID RANGES OR ALL`

Example response:

```
MSER790001TOMSER790025
END
```

4. Display:

```
ENTER RONLY-PLATE COUNT DATA REPORT ONLY
      FONLY-FORTRAN OUTPUT FILE ONLY
      BOTH-GENERATE REPORT AND FORTRAN FILE
```

Response: Enter only one of: RONLY, FONLY, BOTH

Note: FONLY provides only for the statistical summary page.

5. Display:

```
NO. OF RECORDS SELECTED  NNNN
NO. OF TESTS REPORTED    NNNN
```

6. If you realize that you have made an inappropriate response and wish to start over again, do the following:

Hit the BREAK key

Enter:

```
@@X TIO
@ADD RUNS.ASSAY40
```

7. If you wish to have a printed copy of the plate count report:

Enter: `@ADD RUNS.PRINT40`

8. If you wish to plot the data selected using the TEKTRONIX terminal and hard copier:

Enter: `@ADD RUNS.PLOT`

Wait and plots will be printed on your TEKTRONIX hard copier.

NOTE: This is a very expensive alternative and should be used only when absolutely necessary.

9. If you wish to plot the data off-line using a printer plot:

Enter: `@ADD RUNS.PRINT-PLOT`

Be careful. This run may reach "MAX TIME" if too many plots are requested.

10. After copies received and/or session is completed:

Enter: @FIN

DEMAND MODE: PLATE COUNT SUMMARY REPORT

1. Enter: @ADD RUNS.ASSAY60-NEW

2. Reply to queries as shown below:

Display: ENTER A LAB AND DATE OR A LAB AND DATE RANGE OR ALL IN THE
FORMAT: XXXXMMDDYY OR XXXXMMDDYY-MMDDYY OR ALL

Example response: NSNC011579-031579

Above can be repeated until all selections are entered. Then after the last date:

Enter: END

3. Send report to be printed at main site:

Enter: @ADD RUNS.PRINT60-NEW

4. GRAPH60 provides a graphical summary of the plate count summary report.

Enter: @ADD RUNS.GRAPH60

Program will provide the range of dates available.

Display: ENTER THE DATES, STRAIN, AND SAMPLE ID YOU WANT TO PLOT

Example response: 011579-020179 TA98 NSNC800011-NSNC800015

Program will display data selected.

Display: DO YOU WANT TO CHANGE ANYTHING? (YES OR NO)

Enter: YES (if you need to alter your selections) or
NO (if information is correct).

Display: DO YOU WANT TO PLOT THESE DATA BY STRAIN OR BY SAMPLE ID?
(STRAIN OR SAMPID)

Enter: STRAIN or
SAMPID

4. (continued)

Graphs will now be automatically plotted on the TEKTRONIX terminal and copied on the hard copier.

Display: DO YOU WANT TO CONTINUE? (YES OR NO)

Enter: YES or
NO

BATCH MODE: FORTRAN OUTPUT FILE

1. Enter: @ADD RUNS.40-BATCH

2. Display: ENTER A SINGLE RESEARCH LAB OR ALL:

   Response: Choose and enter one of the four-letter codes from the Research Laboratory Code Sheet (Table A-2), e.g., NEIC.

3. Display: ENTER UP TO 10 SAMPLE IDS OR SAMPLE ID RANGES OR ALL
   ENTER END TO TERMINATE SELECTION

   Example response: EMSL790001TOEMSL790027
   MSER780101TOMSER790005
   MSER770023
   END

4. Display: NO. OF RECORDS SELECTED NNNN
   NO. OF TESTS REPORTED NNNN

5. If you make an inappropriate response and wish to start over, do the following:

   Hit the BREAK key.

   Enter: @@X TIO

   Enter: @ADD RUNS.40-BATCH

6. Enter: @START RUNS.PLOT-BATCH

7. Enter: @FIN

8. Wait for job to finish!

9. Get on system, if needed, as in steps A1 to A7.

10. Enter:

```
ID/PASSWORD
@@SEND,U
```

11. You may have to wait a while here. Then you should start receiving data sheets.

12. To resend:

```
@RUN
@SYM,U PLFILE,,,LTC/U
@FIN
LTC/LTC
@@SEND,U
```

13. Enter: `@FIN`

BATCH MODE: PLATE COUNT SUMMARY REPORT

1. Enter:

```
@ELT,I                   RUNS.SELECT
BMGS                     (LAB WANTED OR ALL)
011279                   (DATE, DATE RANGE OR ALL)
NSNC
011279-021579
END
@END
```

2. Enter:

```
@START RUNS.60-BATCH
@FIN
```

3. Report is picked up in A-wing.

4. To obtain another copy only after first has run:

```
@SYM,U EXSUMRPT,,,PR
```

BATCH MODE: INVENTORY BY SAMPLE ID.

The three options are:

1. Enter:

```
@START RUNS.INVENTORY
@FIN
```

1. (continued)

   This file supplies a list of samples recorded on the system by sample identification number and an indication of which laboratories performed the bioassay.

2. Enter:

   ```
   @START RUNS.INVENTORY2
   @FIN
   ```

   This file supplies sample identification number, sample description, bioassay laboratory, and date tested.

3. Enter:

   ```
   @START RUNS.INVENTORY3
   ```

   This file supplies the information of INVENTORY2 plus listing of tester strains used for testing and whether or not an exogenous activation system was used.

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APPENDIX A

HERL INTERIM *IN VITRO* SYSTEM TABLES

TABLE A-1. SUPPLIER CODES

Code	Supplier
BMGS	Biochemical Microbial Genetics
GBBX	Genetic Bioassay Branch
NSNC	Northrop Services, Inc., North Carolina
NSOS	Northrop Services, Inc., On-Site
SRIC	SRI, California
LITB	Litton Bionetics
EGGM	E G & G Mason
RTIX	Research Triangle Institute, North Carolina
CCCC	Other laboratories
MSDS	Monitoring System Development Section
CASC	Characterization and Assessment Section

TABLE A-2. RESEARCH LABORATORY CODE SHEET

Code	Laboratory/Performing Institution
ALL	All laboratories
BCLO	Battelle-Columbus Laboratories, Ohio
BIOL	Biolabs, Inc., Illinois
BMGS	Biochemical and Microbial Genetics Section
BTLI	Bio-Technics Laboratories, Inc., California
BXCM	Bioassay System Corporation, Maryland
CASC	Characterization and Assessment Section
CCCC	Laboratories not listed in codes
CWRU	Case Western Reserve University, Ohio
EGGM	E G & G Mason Research Institute
GENX	Genex Corporation, Maryland
HLAI	Hazleton Laboratories America, Inc., Virginia
HTRI	Hill Top Research, Inc., Ohio
IITR	IIT Research Institute, Illinois
JPSA	Jefferson Professional Services, Arkansas
LITB	Litton Bionetics, Inc.
LLLN	Litron Laboratories, Ltd., New York
MAMD	Microbiological Associates, Maryland
MLIV	Meloy Laboratory, Inc., Virginia
MRCO	Monsanto Research Corp., Ohio
MSDS	Monitoring System Development Section
NEIC	NEIC, U.S. EPA, Colorado
NSNC	Northrop Services, Inc., North Carolina
NSOS	Northrop Services, Inc., On-site
RSSW	Raltech Scientific Services, Wisconsin
RTIX	Research Triangle Institute
SFRE	Southwest Foundation for Research and Education, Texas
SRIC	SRI International (California)
URCA	UNILAB Research, California

TABLE A-3. SAMPLE PREPARATION LABORATORY CODE

Code	Branch/Division/Group/Section
HERL-RTP-NC	
BMGS	Biochemical and Microbial Genetic Section
CASC	Characterization and Assessment Section
CMBX	Carcinogenesis and Metabolism Branch
EBDX	Experimental Biology Division
ETDX	Experimental Toxicology Division
GBBX	Genetic Bioassay Branch
GTDX	Genetic Toxicology Division
HSDX	Human Studies Division
JOEL	Branch Office, Genetic Bioassay Branch
MSDS	Monitoring System Development Section
EMSL-REP-NC	
FSSX	Field Studies Section
SFMC	Sources, Fuels, and Molecular Chemistry
ESRL-RTP-NC	
ARBX	Aerosol Research Branch
MSER	Mobile Source Emissions Research Branch
IERL-RTP-NC	
APBX	Advanced Process Branch
CRBX	Combustion Research Branch
FPBX	Fuel Process Branch
PTBX	Particulate Technology Branch
CINCINNATI	
CINH	Cincinnati - Health Research Group
ANN ARBOR	
CABS	Characterization and Emission Branch
SDSB	Standards Development and Support Branch
TAEB	Technology Assessment and Evaluation Branch
OTHERS	
CCCC	Other Laboratories
DUKE	Duke University
UNEX	University of North Carolina

TABLE A-4. UNITS TABLE (CONCENTRATION/DENSITY SUBSET)

Description	Abbreviation
Milligrams Per Kilogram	MG/KG
(micrograms per gram)	UG/G
Milligrams Per Gram	MG/GM
(grams per kilogram)	G/KG
Micrograms Per Kilogram	UG/KG

TABLE A-5. UNITS TABLE (MASS SUBSET)

Description	Code
Not Applicable	NA
Not Specified	NS
Other	OT
Teragram	TG
Gigagram	GG
Kilogram	KG
Hectogram	HG
Gram	GM
Decigram	DG
Centigram	CG
Milligram	MG
Microgram	UG
Nanogram	NG
Picogram (micromicrogram)	PG

TABLE A-6. UNITS TABLE (TIME SUBSET)

Description	Abbreviation
Not Applicable	NA
Not Specified	NS
Other	OT
Megasecond	MS
Kilosecond	KS
Hectosecond	HS
Second	SE
Decisecond	DS
Centisecond	CS
Millisecond	MS
Microsecond	US
Nanosecond	NS
Picosecond	PS
Minute	MN
Hour	HR
Day	DA
Week	WK
Year	YR

TABLE A-7. INDUCER CODES

Compound	Code number
None	01
Phenobarbitol	02
3-Methylcholanthrene	04
Aroclor 1254	05
Aroclor 1254 and Phenobarbitol	06
Naphthoflavone	07
Naphthoflavone and Phenobarbitol	08
Other	99

TABLE A-8. ORGAN CODES

Organ	Code number
Liver	01
Lung	02
Kidney	03
Testes	04
Skin	05
Bladder	06
Serum	07
Other	99

TABLE A-9. PREPARATION SOLUTION CODES

Solution	Code number
0.15M Potassium Chloride	01
0.15M Potassium Chloride + 0.001M EDTA	02
0.25M Sucrose	03
Other	99

TABLE A-10. PREPARATION TYPE CODES

Preparation type	Code number
9000 x g supernatant	01
105,000 x g microsomes	02
Whole organ homogenate	03
Other	99

TABLE A-11. METHODS OF HOMOGENIZATION

Homogenization method	Code number
Potter-Elvehjen, by hand	01
Potter-Elvehjen, by stir motor	02
Polytron	03
Waring Blender	04
Other	99

TABLE A-12. ACTIVITY CODES

Technique	Code number
Protein Lowery mg/mL of total protein	01
Aryl hydrocarbon hydroxylase p/mol/min/mg microsomal protein	02
Dimethylnitrosamine demethylase	03
Cytochromes (P448-450 series)	04
Cytochrome P450	05
Cytochrome P448	06
Other	99

TABLE A-13. MICROORGANISM STRAIN CODES

Type of strain	Strain name	Strain code
Salmonella (Ames) Strains	TA1535	TA1535
	TA1537	TA1537
	TA1538	TA1538
	TA100	TA100
	TA98	TA98
	TA90	TA90
	TA92	TA92
	LT2	LT2
	TA1530	TA1530
	TA1532	TA1532
	TA1534	TA1534
	TA1950	TA1950
	TA1952	TA1952
	TA1963	TA1963
	TA1975	TA1975
	TA1977	TA1977
	TA1978	TA1978
	TA2410	TA2410
	TA2631	TA2631
	TA2637	TA2637
	TA2641	TA2641
	G46	G46
	C3076	C3076
	D3052	D3052
Salmonella (Ames) Strains Used for 8AG Resistance	8A1535	8A1535
	8A1537	8A1537
	8A1538	8A1538
	8A100	8A100
	8A98	8A98
Salmonella (Skopek-Thilly) Strains	TM35	TM35
	TM677	TM677

(continued)

TABLE A-13. (continued)

Type of strain	Strain name	Strain code
Salmonella Nitroreductase-Deficient Strains	TA1535ND	1535ND
	TA1537ND	1537ND
	TA1538ND	1538ND
	TA98ND	98ND
	TA100ND	100ND
	TA98/NR	TA98NR
	TA98/1,8DNP$_6$	98P6
	TAoi/NR/1,8DNP$_1$	98NRP1
	TA98/NR/1,8DNP$_2$	98NRP2
	TA98/NR/1,8DNP$_5$	98NRP5
	TA98/1,8DNP$_6$/NR$_1$	98P6N1
	TA98/1,8DNP$_6$/NR$_8$	98P6N8
	TA98/1,8DNP$_6$/NR$_{11}$	98P6NA
	TA98/1,8DNP$_6$/NR$_{12}$	98P6NB
	TA98NIR301	98NIR1
	TA98NIR302	98NIR2
	TA98NIR303	98NIR3
	TA98NIR304	98NIR4
	TA100/NR	100NR
	TA98FR$_1$	98ND
	TA100FR$_1$	100ND

TABLE A-14. TEST TYPE CODES

Test type	Code number
Standard Plate Incorporation	01
Spot Test	02
Plate Test - Gas Chamber	03
Plate Test - Vapor from Spot on Lid	04
Plate Test - Pre-incubation	05
Suspension Test	06
Suspension Test Exposed to Gas	07
Suspension Test - Gas-Bubbled	08
DNA Repair Spot Plate Test	09
DNA Repair Suspension Test	10
Survival Data - Pre-incubation	15
Survival Data - Suspension Test	16
Other	99

TABLE A-15. SOLVENT AND POSITIVE CODES

Codes	Code number
Solvent Codes	
Negative (no solvent)	50
Distilled Water	51
Normal Saline	52
K Phosphate Buffer 0.050-0.10M ph 7.0	53
Dimethyl sulfoxide	54
100 Percent Ethanol	55
Methanol	56
Acetone	57
Corn Oil	58
Other Solvent	99
Positive Codes	
Sodium Azide (Na azide)	01
9-AA	02
2-NF	03
2-AA, 2A, 2-Aminoanthracine	04
MNNG	05
AF-2 (Aflatoxin)	06
2-Aminofluorene (2AF)	07
DMBA	08
Other Positive	09

TABLE A-16. BACKGROUND CODES

Background	Code number
Normal	1, blank
Partially Clear	2
Clear	3
Contaminated	4
Precipitation and Normal	5
Precipitation and Partially Clear	6
Precipitation and Clear	7
Small Colonies	8
Heavy	9
Other A-Z	A to Z

TABLE A-17. PHENOCOPY CHECK CONCLUSION CODES

Phenocopy check conclusion	Code number
True Mutants	1
Phenocopies	2
Mixed Population	3
Questionable	4
Other	9

TABLE A-18. SPECIES-STRAIN CODES

Test animal description	Code number
Not applicable	1
Not specified	2
Other	3
Mice	4
Mice-B6C3F1	5
Mice-Swiss	6
Mice-C57/BL6	7
Mice/BALB/C	8
Mice-C57/BL/6J	9
Mice-C3H/Hen MTV+	10
Mice-C3H/Hen MTV-	11
Mice-F1	12
Mice-F2	13
Mice-C3H/HEJ MTV+	14
Mice-C3HEB/FEJ MTV-	15
Mice-BA (F1)	16
Mice-HC (F1)	17
Mice-BA,HC (F2)	18
Mice-HC,BA (F2)	19
Mice-CRL: CD-1	20
Mice-A/J	21
Mice-C3H/HEJ	22
Mice-DBA/2J	23
Mice-BALB/CJ	24
Mice-C57BL/6J Inbred	25
Mice-CBA/J	26
Mice-C3H	27
Mice-SJL/J	28
Mice-B6D2F1	29
Mice-CFW	30
Mice-NUDE	31
Hamsters	32
Hamsters-Golden Syrian	33

(continued)

TABLE A-18. (continued)

Test animal description	Code number
Guinea Pigs	34
Guinea Pigs-2	35
Guinea Pigs-13	36
Rats	37
Rats-Fischer 344	38
Rats-Sherman	39
Rats-Wistar	40
Rats-Long-Evans	41
Rats-Osborne-Mendel	42
Rats-Sprague-Dawley	43
Rats-August 990	44
Rats-Marshal 545	45
Rats-ACI	46
Rats-SCH:	47
Rats-HUT:	48
Rats-CRL:	49
Rats-Fischer	50
Rats-SP-DF	51
Rats-CD	52
Dogs	53
Dog-Beagle	54
Rabbits	55
Rabbit-New Zealand White	56
Goat	57
Goat-Angora	58
Drosophila melanogaster Oregon-R	59
Drosophila melanogaster Canton-S	60
Drosophila melanogaster Urbana-S	61
Drosophila melanogaster Lousanne-S	62

TABLE A-19. ROUTES OF ADMINISTRATION CODES

Route	Code number
Not applicable	1
Not specified	2
Other	3
Oral, Food	4
Oral, Water	5
Oral, Tablet	6
Oral, Capsule	7
Oral, Gavage	8
Injection, Subcutaneous	9
Injection, Intramuscular	10
Injection, Intravenous	11
Injection, Intraperitoneal	12
Implant, Subcutaneous	13
Implant, Bladder	14
Implant, Organ	15
Implant, Tissue	16
Instillation, Intratracheal	17
Instillation, Nasal	18
Instillation, Conjunctival	19
Instillation, Bladder	20
Ocular, Dry Powder	21
Ocular, Liquid	22
Dermal, Solution	23
Dermal, Dry Powder	24
Dermal, Wet Powder	25
Respiratory Exposure, Total Body	26
Respiratory Exposure, Hood or Mask	27
Perfusion	28
In utero	29
Combined Exposure (Total Body)	30
Exposure Following Other Treatment	31

APPENDIX B

QUALITY ASSURANCE QUESTIONNAIRE

In order to provide for on-going quality assurance/quality control measures within the Genetic Toxicology Division's and contractors' laboratories, the following questionnaire and checklist have been constructed to evaluate, in a qualitative manner, each microbial testing laboratory.

QUALITY ASSURANCE INFORMATION FOR SALMONELLA TYPHIMURIUM BIOASSAYS

After each question, please place an "X" in the box with the appropriate answer for that question. Or, if appropriate, fill in the blank after the question. These forms are submitted only upon the request of the EPA project officer and need not be submitted for each report.

1. Is the Ames Protocol (1975), as modified by deSerres and Shelby (1979), followed?

Yes
No

2. How is the initial toxicity range normally determined for use in mutagenicity testing?

Qualitative testing
Quantitative testing

3. What types of test are used?

Pour (Plate incorporation)
Spot
Well
Suspension
Fluctuation
Pre-incubation
Host-mediated
Co-cultivation
Other ____

4. Are protocols for each of the tests used attached?

Yes
No

5. What animal is used routinely in preparing mammalian microsomal enzyme systems?

Rat
Mouse
Hamster
Other (Specify) ____________________________________

6. At time of induction, what animal weight range is allowed?

__________ g to __________ g

6a. Is S-9 usually induced?

Yes
No

7. What are the maximum allowable storage time and storage temperature for S-9?

(a) ___________ days (b) ___________°C

8. How many dosage levels are used generally for the test compound?

___________ levels

9. How many replicate plates are used routinely for each dose?

___________ replicate plates/dose

10. In the mutagenicity test, what range is allowed in the number of viable cells/plate?

___________ to ___________ cells x 10^8

11. What are the incubation time and temperature routinely used for the agar plates?

(a) ___________ hours (b) ___________°C

12. What is the laboratory's spontaneous background range for the following bacterial strains? (Leave spaces blank if a strain is not used.)

a. Without metabolic activation

	Low	High	Mean	Standard Deviation	No. of Experiments
TA1535					
TA1537					
TA1538					
TA98					
TA100					

b. With metabolic activation

	Low	High	Mean	Standard Deviation	No. of Experiments
TA1535					
TA1537					
TA1538					
TA98					
TA100					

c. Over what period of time were values for these ranges compiled?

___________ months

13. Is an entire experiment for each chemical with each strain routinely repeated on a different day from the initial testing?

Yes No

14a. Are any outlier rejection procedures used in analyzing the data?

Yes No

14b. If yes, please describe and give references.

15a. How is a positive mutagenic response determined?

Quantitatively Qualitatively

15b. Please describe criteria used to determine mutagenicity:

16. How many doses are routinely run in an experiment?

___________ doses

17. Are enzyme levels measured for S-9 activity before use?

Yes No

18. Is S-9 evaluated against known positive controls (such as 7,12-dimethylbenzanthracene [DMBA])?

Yes No

19. Are bacterial strains checked for markers (ampicillin resistance, cell wall mutation, UV sensitivity, and histidine sensitivity)?

Yes No

20. Are revertants checked by replating (phenocopy check)?

Yes No

21a. How are plates counted?

By hand　　　　　By machine

21b. Describe the machine and calibration methods used.

22. Are all test components tested separately for sterility?

Yes　　　　　No

REFERENCES

Ames, B.N., J. McCann, and E. Yamasaki. Methods for Detecting Carcinogens and Mutagens with the *Salmonella*/Mammalian-Microsome Mutagenicity Test, Mutation Res. 31:347-364, 1975.

deSerres, F.J., and M. Shelby. Recommendations on Data Production and Analysis using the *Salmonella*/Microsome Mutagenicity Assay, Mutation Res. 64:159-165, 1979.

COMMENTS OR ADDITIONAL INFORMATION:

(Please use additional sheet if necessary.)

AMES/SALMONELLA SYSTEMS AUDIT CHECKLIST

Laboratory: ______________________________

QA Inspection: ______________________________

Date: ______________________________

LABORATORY

( ) Configuration ______________________________
( ) Appearance ______________________________
( ) Organization ______________________________
( ) Cleanliness ______________________________

Equipment:
( ) Shakers ______________________________
( ) Autoclave ______________________________
( ) Washing Facilities ______________________________
( ) Hoods (Laminar Flow and Transfer) ______________________________
( ) Disposal Facilities ______________________________
( ) Automatic Plate Pourer ______________________________
( ) Storage ______________________________
( ) Freezer ______________________________
( ) Colony Counters ______________________________
( ) Animal Facility ______________________________
( ) Incubators ______________________________
( ) Toxic Laboratory ______________________________

( ) Standard Operating Procedure ______________________________

Personnel:
( ) Resumes ______________________________
( ) Education ______________________________
( ) Experience ______________________________
( ) Competence ______________________________
( ) Number/Work ______________________________
( ) Motivation/Attitude ______________________________
( ) Supervisor ______________________________
( ) Chain-of-Command ______________________________
( ) Feedback ______________________________
( ) On-the-Job Technical Training ______________________________

COMMENTS: ______________________________
____
____
____

LEGEND: (SA) = Satisfactory
(U) = Unsatisfactory
(NA) = Not Applicable

(continued)

AMES/SALMONELLA SYSTEMS AUDIT CHECKLIST (continued)

ASSAY

( ) Ames Protocol (1975) as modified by deSerres and Shelby (1979) followed

( ) Toxicity Determination (Quantitative or Qualitative) Type Tests

( ) Dose Levels (No. experiment)
( ) No. Replicate Plates/Dose
( ) Experiment Repeated on Separate Day
( ) Phenocopy Check
( ) No. Viable Cells/Plate
( ) Sterility/Components
( ) Growth Ability/Components

Storage:
( ) Time ( ) Temp.

Spontaneous Background Range (low-high):

	-AM	+AM
TA1535		
TA1537		
TA1537		
TA98		
TA100		

Sample:
( ) Collection
( ) Fractionation
Storage:
( ) Time ( ) Temp.
Genetic Markers Checked:
( ) UVBr
( ) rfa
( ) Ampicillin
( ) Histidine
( ) How Often
Plates Counted:
( ) By Hand
( ) Automatically
( ) How Calibrated
( ) How Often
Solvent QC:
( ) Toxicity
( ) Amt./Plate
( ) S-9 Activity

COMMENTS:

(continued)

S-9

( ) Strain of Animal Used for S-9 ____________________
( ) No./Animals ____________________
( ) Wt./Animals ____________________
( ) mg S-9/Plate ____________________
( ) Activity Checked (method) ____________________
Induction Completed:
- ( ) Type ____________________
- ( ) Amt./Animal ____________________
- ( ) Induction Scheme ____________________

( ) Sterility Checked ____________________
Storage:
- ( ) Time ____________ ( ) Temp. ____________

( ) Enzyme Levels Checked Before Use ____________________
( ) Positive Control Compounds Used to Check Activity ____________________
( ) S-9 Mix Composition ____________________
( ) Cofactors ____________________

COMMENTS: ____________________
____
____
____

DATA

( ) Standard Reporting Form ____________________
( ) Standard Deviation and Variance Calculated ____________________
Positive Mutagenic Response:
- ( ) How Determined (Qualitatively/Quantitatively) ____________________
- ( ) Criteria ____________________
____
____

Standard Deviation:
- ( ) Toxicity ____________________
- ( ) Mutagenicity ____________________
- ( ) Positive Controls ____________________
- ( ) Negative Controls ____________________

Variance:
- ( ) Toxicity ____________________
- ( ) Mutagenicity ____________________
- ( ) Positive Controls ____________________
- ( ) Negative Controls ____________________

( ) Data Reduction Method ____________________
( ) Data Storage Method ____________________
( ) Reproducibility ____________________
( ) Publications ____________________

COMMENTS: ____________________
____
____
____

APPENDIX C

THE COMPLEX SAMPLE EXPANSION CODE

The Complex Sample Expansion Code describes the collection site for a sample in relation to the SIC/Census Bureau code number. This code number is composed of up to four digits. The first digit describes the type of sample, the second digit describes the type of location, the third digit describes a more precise collection site or method, and the fourth digit presents the final type of sample.

The first digit corresponds to the categories listed below:

1 - Air
2 - Water
3 - Land/Soil
4 - Fluids (Other than water)
5 - Solids (Other than Land/Soil)
6 - Vegetation
7 - Animals/Animal tissues
8 - Humans/Human tissues
9 - Other

The following table (Table C-1) presents the expansion of the above categories and thus an explanation of the last three digits in the code. For example, the code number 1145 represents: air (first digit is 1), industrial (second digit is 1), indoor (third digit is 4), and gases tested in situ (fourth digit is 5). Therefore, a gaseous, volatile (not particulate), air sample was taken at an industrial indoor site.

TABLE C-1. COMPLEX SAMPLE EXPANSION CODE

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Air)

1

-1 Industrial
-2 Industrial: Business/Managerial Site
-3 Public
-4 Municipal
-5 Suburban
-6 Rural
-7 Product Sampling
-8
-9 Other

--1 Outdoor Ambient
--2 Indoor Ambient
--3 Outdoor: In Exhaust Plume
--4 Indoor: Proximity of Emission Site
--5 Within/From Emission Control Process
--6 From Attached Dilution/Collector Device(s)
--7 Fugitive Emission Collection
--8
--9 Other

---1 Total: In situ
---2 Total: Collected
---3 Particulate: In situ
---4 Particulate: Collected
---5 Gases/Volatiles: In situ
---6 Gases/Volatiles: Collected
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Water)

2

-1 Industrial
-2 Industrial: Business/Managerial Site
-3 Public
-4 Municipal
-5 Suburban
-6 Rural
-7 Product Sampling
-8
-9 Other

--1 Drinking/Tap
--2 Industrial Use (Including Recycled)
--3 Lake/River
--4 Small Stream/Pond
--5 Ocean/Sea (Marine)
--6 Within/From Emission Control Process
--7 Springs, Wells, Underground Sources
--8
--9 Other

---1 Total Grab Bag
---2 Total Select Sampling Methods
---3 Filtration Collection (Particles, Filters, Columns, etc.)
---4 Sludge/Slurry (Bottom Collection)
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product
(Matrix) and Expansion of Code Numbers

(Land)

3

-1 Industrial
-2 Industrial: Business/Managerial Site
-3 Public
-4 Municipal
-5 Suburban
-6 Rural
-7 Product Sampling
-8
-9 Other

--1 Agricultural Use
--2 Natural/Forest/Nondeveloped Use
--3 Esthetic Use (Gardens, Lawns, etc.)
--4 Industrial/Commercial (Storage, Parking, etc.)
--5 Waste Storage/Disposal
--6
--7
--8
--9 Other

---1 Total/Mixed Soil Sample
---2 Specific Layer Sampling
---3 Leachate Sample
---4
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Fluids - Other than water)

4

-1 Industrial
-2 Industrial: Business/Managerial Site
-3 Public
-4 Municipal
-5 Suburban
-6 Rural
-7 Product Sampling (e.g., Industrial Mixtures)
-8
-9 Other

--1 Outdoor Industrial Storage
--2 Indoor Industrial Storage
--3 Outdoor Spills/Leaks/Emissions
--4 Indoor Spills/Leaks/Emissions
--5 From Product Container
--6 Spent Fluid from Specific Use (e.g., Crankcase Oils)
--7
--8
--9 Other

---1 Total Grab Bag
---2 Total Select Sample Methods
---3 Filtration Collection
---4
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Solids)

5

-1 Industrial
-2 Industrial: Business/Managerial Site
-3 Public
-4 Municipal
-5 Suburban
-6 Rural
-7 Product Sampling
-8
-9 Other

--1 Industrial Raw Use Product
--2 Industrial Formulation
--3 Industrial By-Product
--4 Outdoor Spills
--5 Indoor Spills
--6 Waste Products
--7
--8
--9 Other

---1 Total Grab Bag Sample
---2 Total Select Sample Method
---3 Sized Particles
---4
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Vegetation)

6

-1 Agricultural
-2 Natural
-3 Ornamental
-4 Laboratory
-5
-6
-7
-8
-9 Other

--1 Outdoor
--2 Indoor (Other than Greenhouse)
--3 Greenhouse
--4
--5
--6
--7
--8
--9 Other

---1 Total Plant
---2 Specific Plant Segments
---3
---4
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Animals/Animal Tissues)

7

-1 Agricultural
-2 Non-domesticated
-3 Domesticated
-4 Zoological
-5 Laboratory
-6
-7
-8
-9 Other

--1 Embryonic
--2 Newborn (Not Weaned)
--3 Adult
--4
--5
--6
--7
--8
--9 Other

---1 Body Fluids (Non-invasive)
---2 Body Fluids (Invasive)
---3 Specific Organ/Tissue
---4 Total Animal
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product

(Matrix) and Expansion of Code Numbers

(Human/Human Tissues)

8

-1 Industrial Worker
-2 Managerial/Clerical Workers/Students (Office Jobs)
-3 Domestic Workers
-4 Medical Industry Workers/Laboratory Workers
-5 Accidental Exposure Group
-6 Non-employed
-7 Medically-exposed
-8
-9 Other

--1 Fetal
--2 Infant
--3 Child
--4 Adult
--5 Geriatric
--6 Post-mortem
--7
--8
--9 Other

---1 Non-invasive/Body Fluids
---2 Body Fluids (Invasive)
---3 Specific Organs/Tissues
---4
---5
---6
---7
---8
---9 Other

(continued)

TABLE C-1. (continued)

Collection in Relation to SIC Site/Product (Matrix) and Expansion of Code Numbers
(Other)
9
-1
-2
-3
-4
-5
-6
-7
-8
-9 Other
--1
--2
--3
--4
--5
--6
--7
--8
--9 Other
---1
---2
---3
---4
---5
---6
---7
---8
---9 Other